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(57) Abstract

The present invention generally relates to peptides and compounds which bind to the SH2 domain or domains of various proteins, as well as methods for identifying such peptides and compounds. These peptides and compounds have application as agonists and antagonists of SH2 domain containing proteins, and as diagnostic or therapeutic agents for the diagnosis or treatment of disease conditions.

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PEPTIDES AND COMPOUNDS THAT BIND TO SH2 DOMAINS

FIELD OF THE INVENTION

The present invention generally relates to peptides and compounds which bind to the SH2 domain or domains of various proteins, as well as methods for identifying such peptides and compounds. These peptides and compounds have application as agonists and antagonists of SH2 domain containing proteins, and as diagnostic or therapeutic agents for the diagnosis or treatment of disease conditions.

BACKGROUND OF THE INVENTION

SH2 (src homology region 2) domains are protein motifs consisting of approximately 100 amino acids which were originally described by Sadowski *et al.*, *Mol. Cell Biol.*, **6**:4396-4408 (1986). These domains were discovered when the noncatalytic domains of cytoplasmic tyrosine kinases such as fps were compared to src (Sadowski et al., Id.) and subsequently identified in the enzyme PLC-γ. (*See*, Suh *et al.*, *Proc. Natl. Acad. Sci., USA*; **85**:5419-5423 (1988); Stahl *et al.*, *Nature*, **332**:269-272 (1988) and also to the oncogene v-crk (Mayer *et al.*, *Nature*, **332**:272-275 (1988)). It is also known that many proteins having SH2 domains are involved in pathways downstream of growth factor receptors such as ras GTP-ase activating protein, phosphatidyl-inositol 3'-kinase, and phospholipase C. (*See*, Waksman *et al.*, *Cell*, **72**:779-790 (1993)).

The discovery that SH2 domains bind to sequences containing phosphorylated tyrosine residues has provided a link between tyrosine kinases and proteins that respond to tyrosine phosphorylation. For reviews in this area, *see*, Koch *et al.*, *Science*, **252**:668-674 (1991); Pawson and Gish, *Cell*, **71**:359-362 (1992); and Mayer and Baltimore, *Nature*, **322**:272-275 (1993).

The fact that SH2 domains bind to protein sequences containing phosphotyrosine, coupled with the fact that SH2 domains have been identified in molecules associated with growth factor mediated intracellular signalling and oncogenesis, indicates that these domains are significant in pathways utilized in growth regulation. (*See*, *e.g.*, Ullrich *et al.*, *Cell*, **61**:203-211 (1990); Cantley *et al.*, *Cell*, **64**:281-302 (1991)). Related to this, proteins containing SH2 domains have been implicated in cellular transformation, signal transduction, response to growth factor stimulation, and cellular growth and

differentiation. (*See*, Koch *et al.*, *Science*, **252**:668-674 (1991); Margolis, *Cell Growth and Differentiation*, **3**:73-80 (1992)).

Because SH2 domains are contained in src, it is useful to have an understanding of the src structure, in order to understand SH2 domains. In general, src comprises a tyrosine kinase domain, also known as src homology region 1. Immediately amino-terminal thereto is the SH2 domain, which is referred to by some researchers as the B and C box. (*See*, Stahl *et al.*, *Nature*, **332**:269-272 (1988); and Mayer et al., *Nature*, **332**:272-275 (1988)). Immediately amino-terminal to the SH2 domain is the SH3 domain, also known as the A box (Stahl et al. (Id.) and Mayer et al. (Id.)). SH3 domains are approximately 50 amino acids long. SH3 domains have been identified in various proteins, including cytoskeletal proteins such as spectrin. (Wasenius *et al.*, *J. Cell Biol.*, **108**:79-83 (1989).)

Proteins with SH2 domains may or may not have accompanying SH3 domains. See Koch et al who disclose that there exist a number of proteins which comprise the SH2 and SH3 domain. (Koch *et al.*, *Science*, **252**:668-674 (1991).)

The structures of various SH2 domains have been reported in the literature. For example, SH2 domains have been identified in about 20 different cytosolic proteins, including src, abl, syk, PTP1C, PLC, GAP, SHPTP2, vav, PI3K, c-crk, SHC, nck, ISG-F3, and Sem-5/GRB2. Moreover, the amino acid sequences of different SH2 domains have been compared. (*See*, Koch *et al.*, *Science*, **252**:668-674 (1991).) Based on these comparative studies, it is known that SH2 domains typically comprise about 100 amino acid residues, which comprise five well conserved sequence motifs separated by variable sequence elements. More specifically, SH2 domains contain about 30 highly conserved amino acid residues, with three of these apparently being invariant.

Proteins which contain SH2 domains have been divided into at least two and possibly more different categories. One group consists of proteins with SH2 domains which associate with or comprise enzymatic activity. Examples of this first group of proteins includes by way of example the cytoplasmic tyrosine kinases such as src and fps, PLC-γ, ras GAP (*See*, Vogel *et al.*, *Nature*, **335**:90-93 (1988); Trahey *et al.*, *Science*, **242**:1677-1700 (1988)) and PI-3′ kinase associated p85 (Skolnik *et al.*, *Cell*, **65**:83-90 (1991); Otsu *et al.*, *Cell*, 91-104 (1991); Escobedo *et al.*, *Cell*, **65**:75-82 (1991)).

The second group of SH2 domain containing proteins consists of proteins that comprise only SH2 and SH3 domains with few extraneous (residues not contained in SH2 and SH3 domains) amino acid residues. This includes proteins such as crk (Mayer *et al.*, *Nature*, **332**:272-275 (1988), and nck (Lehmann *et al.*, *Nucl. Acids Res.*, **18**:1048 (1990)).

The third group of SH2 domain proteins includes proteins which, based on their structure and/or activity, do not fit into the first two groups. Examples of these proteins include vav (Katzav *et al.*, *EMBO J.*, **8**:2283-2290 (1990)) and tensin (Davis *et al.*, *Science*, **252**:712-715 (1991)). However, it is probable that this third classification will change, and possibly the other two groups as well, as more SH2 domain proteins become identified and their specific biological functions become known.

One SH2 domain containing protein that has been the subject of much recent study is the Sem-5 protein, largely because this protein has been implicated in the signalling pathway from tyrosine kinase receptors to ras oncoproteins, which signalling activates the ras family of proto-oncogenes. Sem-5 is a small protein in *C. elegans* consisting entirely of two SH3 domains flanking an SH2 domain. Mutations in sem-5 can be rescued by activated ras alleles indicating that this protein functions upstream of the let-60 gene, another gene which like Sem-5 is believed to be a component of the ras pathway to transduce growth and differentiation signals. (Clark *et al.*, *Nature*, **3561-9**:340-344 (1992).)

Sem-5 is believed to function as an adaptor which provides for the assembly of other proteins into multi-protein complexes. (Clark *et al.*, *Nature*, **356**:340-344 (1992); Pawson *et al.*, *Nature*, **356**:285-286 (1992).) This is believed to be effected by means of SH3 and SH2 domains contained therein which bind to short proline-rich sequence motifs and phosphotyrosine-containing peptides respectively. This, moreover, is consistent with the observation that SH2 domains, which are found in various effector proteins, have been implicated in the recruitment of these effector proteins into complexes with autophosphylated tyrosine kinase receptors. (Pawson *et al.*, *Cell*, **71**:359-362 (1992).) A Drosophila homologue has also been identified (drk).

In mammals, ras guanine nucleotide exchange activity is stimulated by tyrosine kinase receptors, including the trk-encoded nerve growth factor receptor, the epidermal growth factor receptor (EGFR) and the insulin receptor. A mammalian homologue of

Sem-5, designated Grb2 has also been cloned in screens both for proteins which bind to the autophosphylated EGFR cytoplasmic tail (Lowenstein *et al.*, *Cell*, **13**:155-162 (1993)) and for SH2-containing proteins (Matsuuski *et al.*, *Proc. Natl. Acad. Sci., USA*, **89**:9015-9019 (1992)). A Drosophila homologue has also been identified (drk).

Recently, different research groups have described cell elements by which signals are transmitted from a receptor tyrosine kinase to ras through specific protein-protein interactions. (*See*, Egan *et al.*, *Nature*, **363**:45-51 (1993); Li *et al.*, *Nature*, **363**:85-88 (1993); Gale *et al.*, *Nature*, **363**:88-92 (1993); Rozaksis-Adcock *et al.*, *Nature*, **363**:85-93 (1993); Simon *et al.*, *Cell*, **73**:169-177 (1993); Oliver *et al.*, *Cell*, **73**:179-191 (1993); and Buday *et al.*, *Cell*, **73**:611-62 (1993).) Essentially, these papers all teach that tyrosine kinase receptor activation results in autophosphorylation, which creates a binding site for Grb2, and that Grb2 associates with another protein, Sos1, and recruits it to the activated receptor in the plasma membrane where ras activation is believed to take place.

For example, the SH2 domain of Grb2 binds activated, autophosphorylated EGFR. For other growth factor receptors, *e.g.*, platelet-derived growth factor (PDGF) receptor, another SH2-containing protein, SHC serves as the adaptor linking Grb2 to the activated receptor. Bound adaptor proteins then interact with other cytosolic molecules to induce ras activation. (*See*, Egan *et al.*, *Nature*, **363**:45-51 (1993); Lowenstein *et al.*, *Cell*, **61**:203-212 (1992).)

It has been reported that Sem-5/Grb2/drk selects primarily on the basis of an asparagine residue, two positions from the phosphotyrosine. One example is at position 1068 of EGFR. (Songyang *et al.*, *Cell*, **72**:767-778 (1993).) Recent studies have further indicated that the binding of a particular SH2 domain to a tyrosine-phosphorylated protein is dependent upon the primary amino acid sequence surrounding the phosphotyrosine residue. (*See*, Cantley *et al.*, *Cell*, **64**:281-302 (1991).) For example, sequence comparison of regions of the polyoma middle T and platelet derived growth factor receptor (PDGFR) which bind phosphatidylinisitol sequence pY-Met/Val-X-Met is essential for binding to this protein (Cohen *et al.*, *Proc. Natl. Acad. Sci., USA*, **87**:4458-4462 (1990). Also, this sequence has been useful in predicting other receptors or receptor substrates that bind phosphatidyl-inositol 3-kinase. *See*, *e.g.*, Lev *et al.*, *Proc. Natl. Acad. Sci., USA*, **89**:678-682 (1992). Synthetic phosphopeptides based on this sequence have been found to block phosphatidyl-inositol 3-kinase binding to the PDGF-

receptor and to polyoma middle T. (*See*, Escobedo *et al.*, *Mol. Cell Biol.*, **11**:1125-1132 (1991); and Auger *et al.*, *J. Biol. Chem.*, **267**:5408-5415 (1992).)

Also, Songyang *et al.* reported construction of a phosphopeptide library based on the sequence GDGpYXXXSPLLL, which was screened against the SH2 domains of phosphatidylinisitol 3-kinase, the N- and C-terminal SH2 domains of p85, src, fyn, lck, fyr, abl, crk, nck, Sem-5, the N- and C-terminal domains of PLC-γ and SHPT2N. (*Cell*, **72**:767-778 (1993).) It was reported based on these results that SH2 domains may be categorized based upon the preferred sequences to which they bind. For example, one group (src, fyn, lck, fyr, abl, crl and nck) preferably binds sequences having the general motif pY-hydrophilic-hydrophilic-Ile/Pro, while another group (p85, phospholipase C-γ and SHPTP2) bind the general motif Y-hydrophobic-X-hydrophobic.

References which describe compounds purported to function as SH2 domain protein inhibitors and/or to bind thereto include Burke *et al.*, *Biochemistry*, **33**:6490-6494 (1994); Burke *et al.*, *J. Med. Chem.*, **34**:1577-1581 (1991); Domchek *et al.*, *Biochem.*, 31, 9865-9870 (1992); Songyang *et al.*, *Cell*, **72**:767-778 (1993); WO 94/07913 by Dobrusin *et al.*, published on April 14, 1994; WO 94/03432 by Burke *et al.*, published on February 17, 1994; and WO 94/11392 by Dobrusin *et al.*, published on May 26, 1994.

The recent availability of cloned genes encoding proteins containing SH2 domains should further facilitate the search for antagonists and agonists of SH2 domains. For example, the availability of recombinant proteins containing SH2 domains allows the study of receptor-ligand interactions in a variety of random and semi-random peptide diversity generation systems.

Peptide diversity generation systems include by way of example the "peptides on plasmids" system described in U.S. Patent No. 5,270,170; the "peptides on phage" system described in U.S. Patent Application Serial No. 718,577 filed June 20, 1991 and in Cwirla *et al.*, *Proc. Natl. Acad. Sci., USA*, **87**:6378-6382 (1990), and the "very large scale immobilized polymer synthesis" system (VLSIPS) described in U.S. Patent No. 5,143,854; PCT Patent Publication No. 90/15070, published December 13, 1990; U.S. Patent Application Serial No. 624,120 filed on December 6, 1990; Fodor *et al.*, *Science*, **251**:767-773 (1991); Dower and Fodor, *Ann. Rep. Med. Chem.*, **26**:271-280 (1991), and U.S. Patent Application Serial No. 805,727, filed December 6, 1991. Peptide diversity

generation systems further include the encoded synthetic library (ESL) technology described in copending U.S. Patent Application Serial No. 07/946,231, filed September 16, 1992 and PCT Patent Publication No. 92/00091. Each of the above-identified patents, patent applications, and publications is incorporated by reference herein in its entirety.

However, notwithstanding what is known about SH2 domain containing proteins and their corresponding ligands, there still exists a great need in the art for identifying novel peptides and compounds which specifically bind to SH2 domains of various proteins, including in particular Grb2, given the apparent role of these proteins in the cellular signalling pathway and of Grb2 specifically in the cellular signalling pathway that activates the ras family of proto-oncogenes. The availability of novel peptides and compounds which specifically bind to SH2 domains, preferably with high affinity, will facilitate greater understanding of the specific ligand binding interactions which are involved in cellular signalling as well as other cell responses. Moreover, the identification of novel peptides which bind SH2 domains should further enable the design of nucleic acid probes for identifying ligands which bind to SH2 domain containing proteins. Also, such peptides and compounds will be useful as agonists and antagonists of SH2 domain containing proteins, and therefore have utility for controlling cellular transformation, signal transduction, cellular response to growth factor stimulation, and cellular growth and differentiation, all of which are apparently affected by SH2 domain proteins.

SUMMARY OF THE INVENTION

The present invention provides novel peptides and compounds which bind proteins having an SH2 domain, and particularly which bind to the SH2 domain of Grb2. Preferably, the peptides and compounds will bind to SH2 domain-containing proteins with a binding affinity of less than $1x10^{-5}$ M.

The present invention further provides novel probes for identifying SH2 domain binding proteins and assays for measuring the affinity of test compounds to specific SH2 domains to determine the specificity or selectivity of a particular test compound. Preferably, such specificity targets will include Grb2, c-src, PLCγ, SHC and GAP.

Still further, the present invention provides methods to determine the minimum peptide unit necessary for binding SH2 domains with high affinity, *e.g.*, the SH2 domain of Grb2.

In another aspect, the present invention uses encoded synthetic library (ESL) technology to enable affinity discrimination among a population of beads to which are attached unique peptides having different affinities to an SH2 domain probe.

The present inventon also identifies peptides or compounds having affinity for several SH2 domains as well as peptides which are selective for a specific target SH2 domain.

The present invention still further provides methods of identifying specific modifications of peptides also referred to herein as peptide analogs or peptide mimetics, having enhanced SH2 domain binding properties, *e.g.*, by cyclization or phosphorylation of SH2 domain binding peptides, incorporation of isosteres, *e.g.*, asparagine and/or tyrosine/phosphotyrosine isosteres and constrainment of specific amino acid residues.

In another aspect, the present invention provides methods to improve the metabolic stability and/or cell permeability of SH2 domain binding compounds by the substitution of phosphotyrosine group(s) contained therein with phosphotyrosine/tyrosine isosteres or analogs which lack tyrosine or phosphotyrosine.

In yet another aspect, the present invention provides novel SH2 domain binding peptides and compounds which contain asparagine isosteres.

In still another aspect, the present invention provides methods for the affinity purification of SH2 domain proteins using the novel SH2 domain binding peptides and compounds provided herein.

In still another aspect, the present invention provides methods of using the novel SH2 domain binding peptides and compounds provided herein as antagonists and agonists of SH2 domain containing proteins. More particularly, the methods use these peptides and compounds diagnostically to assay whether SH2 domain proteins are present in a sample, and therapeutically to inhibit or potentiate the activity of an SH2 domain protein.

More particularly, the present invention provides methods of using the novel SH2 domain binding peptides and compounds provided herein for diagnosis, treatment and/or prevention of cancer given the involvement of SH2 domain proteins in oncogenesis and

transformation and of Grb2 specifically in the activation of the ras family of proto-oncogenes.

The present invention also provides a minimal core trimeric sequence, pY-X-N, which provides for adequate binding to the Grb2 SH2 domain, as well as specific peptides containing this core sequence. Related to this, another aspect of the invention is to identify specific amino acids which, when proximate to the core pY-X-N sequence, result in peptides having desirable properties, *e.g.*, binding affinity, selectivity to specific SH2 domains, stability, etc.

The present invention also provides methods for treating diseases associated with aberrant cell growth, differentiation or regulation which are associated with defects in receptor tyrosine kinase pathways utilizing the novel compounds of the invention. The present invention further provides pharmaceutical compositions comprising one or more compounds of the invention and a physiologically acceptable carrier.

The present invention also provides novel libraries which are useful for identifying ligands capable of binding to SH2 domains preferably having a binding affinity of less than about 1×10^{-5} M. These libraries include those containing asparagine and/or phosphotyrosine isosteres, phosphotyrosine "walk" libraries, residues which provide for cyclization, etc. The SH2 domain can, *e.g.*, be selected from the group consisting of src, abl, syk, PTP1C, PLC, GAP, vav, p85, pI-3 K, c-crk, SHC, nck, ISGF3, Sem-5 and Grb2.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts a schematic representation of experiments involving expression of SH2 domain-bacterial fusion proteins. The top of each section shows a representation of the parental protein. Below is shown the specific SH2 domain containing portions of the protein used in these studies.

Figure 2 depicts schematically the MBP and GST vectors for synthesis of SH2 domain containing fusion proteins.

Figure 3 depicts a phage ELISA experiment which measures the target specificity of peptides on Grb2-specific phage which were tested with 3 alternative SH2 domain targets, shc, v-src and PLC.

Figure 4 depicts phage ELISA picks from the second round of panning on the custom YXN library under normal elution conditions (clones 88 through 93) and with peptide elution (clones 94-99).

Figures 5 and **6** depict schematically two alternative peptide detection assays comprising a peptide on a bead, and either MBP-Grb2-179 fusion protein, and a Cy3-anti-AB179 IgG or a GST-Grb2 fusion protein ("double sandwich") protein, and a phycoerythrin-goat anti-rabbit ("triple sandwich").

Figure 7 identifies representative X_1, X_2 and X_3 sequences from the gate 1 beads which exhibited the highest fluorescence and their respective IC_{50}'s, as determined in the radiolabel phosphopeptide competition assay.

Figure 8 depicts results of FACS analysis of peptides on beads showing loss of fluorescence enhanced by peptide competition. In the figure, peptide was added at the arrow symbol, filled symbols have peptide, open symbols lack peptide.

Figure 9 shows the results of an FACS analysis of peptides-on-beads. In the figure, dissociation is shown in the absence (upper) or presence (lower) of FLPVPEpYINQSVP-NH_2 during an overnight incubation.

Figure 10 illustrates tyrosine and phosphotyrosine replacements for use in peptide synthesis.

Figure 11 shows asparagine replacements for use in peptide synthesis.

Figure 12 depicts representative amino acids for ESL constructions.

Figures 13(a-i) depict groups of amino acids which were used in ESL and VLSIPS based peptide construction.

Figure 14 provides the structures of nine monomers used in preparing some peptides of the present invention.

Figures 15-29, 31-37, and 40-45 provide reaction schemes correlating to methods in the Examples.

Figure 30 provides structures for compounds prepared according to the methods in Example 17.

Figures 38 and 39 provide design and synthesis schemes for a number of conformationally-restricted phosphotyrosine and asparagine derivatives.

Figure 46 provides the structures of untagged combinatorial libraries using unnatural amino acids, described in Example 34.

Figures 47(a-f) contains structures and Fmoc numbers for untagged peptide libraries containing B-X-X-X-S-V-PEG15-T and X-N-X-S-V-PEG15-T sequences.

Figures 48(a-s) show representative bead-based libraries of peptides which were prepared.

Figure 49 depicts monomers **A-Z** and **AA-SS** which were used in construction of ESL libraries.

Figure 50 provides the structures of one family of Asn and Asn isosteres which were used in preparation of bead-based libraries.

Figure 51 provides the structures of one family of α,α-disubstituted amino acids which were used in preparation of bead-based libraries.

Figure 52 provides the structures of one family of neutral H-bonding amino acids which were used in preparation of bead-based libraries.

Figure 53 provides the structures of one family of phosphotyrosine isosteres which were used in preparation of bead-based libraries.

Figure 54 provides the structures for a series of tetrapeptides prepared using four different phosphotyrosine isosteres.

Figure 55 provides the structures for a family of peptides prepared on PAL resin.

Figure 56 provides the structures for a family of cyclic peptides prepared as described in Example 39.

Figure 57 shows the results of a peptide binding assay for the phosphopeptide FLPVEpYINQSVP to the Grb2 SH2 domain which shows percent specific binding versus log of competitor concentration.

Figure 58 shows the results of a Grb2 SH2 domain competitive assay between peptides with specificities for the SH2 domains of SH2, Src, GAP and PLC showing total binding (Cpm) versus competitor concentration (μM).

Figure 59 identifies the structures at X_{+1} in tripeptides of the format PTI-X_{+1}-N that bind to Grb2 SH2.

Figure 60 illustrates a fluorescence histogram of a mixture of samples under normal and discriminating conditions.

DETAILED DESCRIPTION OF THE INVENTION

Table of Contents

I. Definitions

Unless otherwise stated, the following terms used in the specification and claims have the meanings given below.

As discussed *supra*, the present invention generally relates to novel SH2 domain binding peptides and compounds, and methods for their identification. In describing this invention, unless defined otherwise all technical and scientific terms have their conventional meanings, as commonly understood by one of ordinary skill in the art. Although other methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described *infra*. Also, to facilitate understanding of the present invention, the following terms are defined below:

"Analog," when used to refer to peptides, refers to polypeptides containing a segment of at least about 3 amino acids having substantial identity to at least a portion of a naturally or non-naturally occurring SH2-binding peptide which exhibits one or both of the following general properties:

(1) specifically binds to at least one SH2 domain; and

(2) effects or blocks an SH2-mediated phenotype.

Typically, analog polypeptides will comprise a conservative amino acid substitution (or addition or deletion) relative to the naturally or non-naturally occurring SH2 domain binding sequences. Analogs typically are at least 5 amino acids long. Preferably, such peptides will be at least as long as the minimal length SH2-binding sequence identified by the methods provided herein for identifying SH2-binding peptides. Analogs may or may not have biological activity. Analogs which lack biological activity

(as well as those possessing biological activity) may be used to affinity purify SH2 domain proteins, as agonists and/or antagonists, both competitive and non-competitive of SH2 proteins, for preparation of antibodies to predetermined epitopes in SH2 domain binding proteins, and as immunological reagents to detect and/or immunopurify antibodies which specifically bind SH2 domain binding proteins.

"Homologous" or "Sequence Identity" in this application is meant to refer to two proteins or polypeptides which share a majority of their amino acid sequences. Generally, this will be at least 90% and usually more than about 95%.

"Label" or "labeled" refers to the incorporation of a detectable marker, *e.g.*, by incorporation of a radiolabeled amino acid or attachment of one or more biotinyl moieties to a polypeptide (wherein attached biotin moieties can be detected using avidin or streptavidin, *e.g.*, streptavidin containing a fluorescent marker or moiety which provides for enzymatic activity which is detectable by optical or colorimetric means). Methods for labeling polypeptides and glycoproteins, as well as other compounds, are well known in the art.

The choice of label is largely an arbitrary design selection and is not essential to the invention. Examples of labels which may be used to label polypeptides include by way of example, radioisotopes (^{3}H, ^{14}C, ^{35}S, ^{125}I, ^{131}I, etc.), fluorescent labels (*e.g.*, RITC, rhodamine, lanthanide, phosphors), enzymes (horseradish peroxidase, β-galactosidase, luciferase, alkaline phosphatase, etc.), biotin and biotin analogs, predetermined polypeptide epitopes recognized by a secondary reporter (*e.g.*, leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitopic tags (*e.g.*, Flag™ octapeptide)). These labels may be directly or indirectly bound, *e.g.*, by covalent attachment of spacers of various lengths, *e.g.*, to reduce or obviate steric hindrance.

"Lower alkyl ester derivative" refers to straight and branched chain alkyl ester derivatives having from about 1 to 6 carbon atoms, *e.g.*, methyl, ethyl, n-propyl, iso-propyl, n-butyl, tert-butyl, n-pentyl, n-hexyl, 1,2-dimethylbutyl ester derivatives, and the like. In general, the lower alkyl ester derivative will be a methyl ester derivative or an ethyl ester derivative.

"Peptide" or "polypeptide" refers to macromolecules which contain two or more amino or imino acids (or their equivalents) in peptide linkage, wherein such peptides

comprise or lack post-translational modification (*e.g.*, glycosylation, cleavage, phosphorylation, side-chain derivation, and the like).

"Pharmaceutically acceptable salts" generally refer to non-toxic alkali metal, alkaline earth metal, and ammonium salts commonly used in the pharmaceutical industry including sodium, potassium, lithium, calcium, magnesium, barium, ammonium and protamine zinc salts. Methods for making such salts are well known in the art. Also, pharmaceutically acceptable salts include non-toxic acid additive salts, which are generally prepared by reacting the compounds of the invention with a suitable organic or inorganic acid. Representative salts include by way of example hydrochloride, hydrobromide, sulfate, bisulfate, acetate, oxalate, valerate, oleate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napsylate, and the like.

"Pharmaceutically acceptable acid addition salt" refers to those salts which retain the biological effectiveness and properties of the free bases and which are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, menthanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. For a description of pharmaceutically acceptable acid addition salts as prodrugs, *see* Bundgaard, H., ed., DESIGN OF PRODRUGS, Elsevier Science Publishers, Amsterdam (1985).

"Pharmaceutically acceptable ester" refers to those esters which retain, upon hydrolysis of the ester bond, the biological effectiveness and properties of the carboxylic acid or alcohol and are not biologically or otherwise undesirable. For a description of pharmaceutically acceptable esters as prodrugs, *see* Bundgaard, H., Id.. These esters are typically formed from the corresponding carboxylic acid and an alcohol. Generally, ester formation can be accomplished via conventional synthetic techniques. (*See*, *e.g.*, March ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York p. 1157 (1985) and references cited therein, and Mark *et al.*, ENCYCLOPEDIA OF CHEMICAL TECHNOLOGY, John Wiley & Sons, New York (1980).) The alcohol component of the ester will generally comprise (i) a C_2-C_{12} aliphatic alcohol that can or cannot contain one

or more double bonds and can or cannot contain branched carbon chains or (ii) a C_7-C_{12} aromatic or heteroaromatic alcohols. Preferably, the ester will comprise a lower alkyl ester. This invention also contemplates the use of those compositions which are both esters as described herein and at the same time are the pharmaceutically acceptable acid addition salts thereof.

"Pharmaceutically acceptable amide" refers to those amides which retain, upon hydrolysis of the amide bond, the biological effectiveness and properties of the carboxylic acid or amine and are not biologically or otherwise undesirable. For a description of pharmaceutically acceptable amides as prodrugs, *see* Bundgaard, H., ed., DESIGN OF PRODRUGS, Elsevier Science Publishers, Amsterdam (1985). These amides are typically formed from the corresponding carboxylic acid and an amine. Generally, amide formation can be accomplished via conventional synthetic techniques. (*See*, *e.g.*, March ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York p. 1152 (1985) and Mark *et al.*, ENCYCLOPEDIA OF CHEMICAL TECHNOLOGY, John Wiley & Sons, New York (1980).) This invention also contemplates the use of those compositions which are both amides as described herein and at the same time are the pharmaceutically acceptable acid addition salts thereof.

"Pharmaceutically or therapeutically acceptable carrier" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to a treated mammal, *e.g.* human patient.

"Stereoisomer" refers to a chemical compound having the same molecular weight, chemical composition, and constitution as another, but with the atoms grouped differently. That is, certain identical chemical moieties are at different orientations in space and, therefore, when pure, has the ability to rotate the plane of polarized light. However, some pure stereoisomers may have an optical rotation that is so slight that it is undetectable with present instrumentation. The compounds of the present invention may have one or more asymmetrical carbon atoms and therefore include various stereoisomers. All stereoisomers are included within the scope of the invention.

"SH2 binding peptide" refers to a polypeptide that binds to a predetermined SH2 domain with a significant binding affinity in suitable aqueous binding conditions (*e.g.*, physiological conditions; 1 x PBS at 37°C). Significant binding affinity, expressed as an IC_{50} in the binding assay described herein, is less than about 1 x 10^{-4} M, usually less than

about 1 x 10^{-5} M, often less than about 1 x 10^{-6} M, preferably less than about 1 x 10^{-7} M. SH2 binding peptides generally possess a biological activity (*e.g.*, biochemical response, specific gene expression, cytoarchitectural change) that is mediated by the predetermined SH2 domain. In some cases, the SH2 binding peptide will block a biological activity by antagonizing the effect of an agonist (i.e., the physiological ligand) for the SH2 domain of the particular protein.

"Biological activity" includes specific binding affinity for ligands, may also include the ability to block the binding of other compounds or ligand, to stimulate or inhibit metabolic pathways, to act as a signal or messenger, to stimulate or inhibit cellular activity and the like, and includes detectable changes in gene expression, biochemical or metabolic pathways, cell architecture, or other detectable cell phenotype that is modulated by the predetermined SH2 domain.

"Substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT (UWGCG Software Package Version 7.0, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA) using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity). Preferably residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine, a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acid substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

"Substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition) and preferably a substantially purified fraction has a composition wherein

the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

"Therapeutically- or pharmaceutically-effective amount" as applied to the compositions of the instant invention refers to the amount of composition sufficient to induce a desired biological result. That result can be alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. In the present invention, the result will typically involve a decrease in the immunological and/or inflammatory responses to infection or tissue injury.

"Diagnostically effective amount" as applied to the compositions of the present invention refers to the amount of the SH2 domain binding peptide or compound necessary to assay the presence of a ligand in a sample, *e.g.*, a SH2 domain polypeptide or an antibody specific to the peptide or compound contained in the particular composition.

Amino acid residues in peptides described in this application will in general be abbreviated using the standard one letter abbreviation for amino acids. Specifically, Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G. In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention.

II. General

Turning now to the specific methods used in the present invention, in general novel peptides, designated "lead" peptides which bind to SH2 domains, *e.g.*, the SH2

domain of Grb2, will be identified by random peptide diversity generating systems. Once such "lead" peptides have been identified, "derivative" compounds are then constructed which possess the same or similar structure as the lead compound, but which differ from the lead compound, *e.g.*, by susceptibility to hydrolysis and/or proteolysis, and/or with respect to other biological properties, such as increased affinity and/or selectivity for specific SH2 domains. Preferred means of derivatization of lead peptides include one or more of the following modifications: cyclization, addition, deletion or substitution of one or more amino acid residues, phosphorylation, addition or substitution of one or more phosphotyrosine and/or asparagine isosteres, and derivatization which results in local constraint of the tyrosine/phosphotyrosine or asparagine moiety.

III. Embodiments of the Invention

A. Peptides That Bind to SH2 Domains

1. Identification by RPD Technology

Lead peptide compounds were identified using random peptide diversity generating systems including the "peptides on phage" system referenced *supra*, and described in U.S. Patent No. 5,270,170 and copending U.S. Application Serial No. 718,577 and No. 07/847,567, filed March 15, 1992.

In general, random peptides were designed to be eight to twelve amino acids in length. Moreover, one system employed fixed cysteine residues at each end of the random peptide sequence to facilitate the formation of cyclic peptides. In order to generate the collection of oligonucleotides that encode the random peptides, the codon motif (NNK)x, when N is a nucleotide selected from A, C, G or T (equimolar, depending on methodology employed, other nucleotides may be employed), K is G or T (equimolar) and x was 8 (for the cyclic library, the other two terminal codons were cysteine codons), 10 or 12; was used in the synthesis of oligonucleotides. Those of ordinary skill in the art understand that the NNK motif encodes all the amino acids, encodes only one stop codon, and reduces codon bias. There are 32 possible codons embraced by the NNK motif, 1 for each of 12 amino acids, 2 for each of 5 amino acids, 3 for each of 3 amino acids, and only one for the three stop codons.

Initially, it was decided to present the random peptides as part of a fusion protein containing either the pIII or pVIII coat protein of a phage fd derivative (peptides on

phage) or the lac repressor DNA binding protein (peptides on plasmids). The fusion proteins, along with the DNA encoding the fusion protein, were then "panned" with an immobilized SH2 domain. This panning process comprises multiple rounds of incubating the expressed fusion proteins that bind to the SH2 domain (along with the accompanying DNA), and amplifying the phage bearing the fusion proteins collected.

In general, after three rounds of panning, the fusion proteins and accompanying DNA are isolated and cultured to produce fusion protein preparations for an ELISA, to determine whether the fusion protein binds specifically to the SH2 domain. This assay was conducted similarly to the panning procedure, except that after removal of unbound fusion proteins, the wells are treated with rabbit anti-phage antibody (or with anti-lac antibody for the peptides on plasmids system), then incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody, and the amount of alkaline phosphatase in each well assayed by standard methods.

By comparing the test well with control wells (no SH2 domain or a different SH2 domain), one can determine whether the fusion proteins bind specifically to the SH2 domain. Fusion proteins that bind specifically to the SH2 domain, are then tested in an SH2 domain blocking assay. This assay is effected similarly to the ELISA, except that the fusion donor protein (*e.g.*, GST) is added to the wells before the fusion protein (the control wells were of two types; (1) no SH2 domain peptide; and (2) no fusion donor protein).

When using random peptide generating systems that allow for multivalent ligand-SH2 domain interaction, the density of the immobilized SH2 domain is significant in determining the avidity of a specific ligand to the immobilized SH2 domain. At higher SH2 domain densities, multivalent binding is more likely to occur (if it occurs at all), than at lower SH2 domain densities (*e.g.*, a lower density comprises each anti-SH2 domain antibody-coated well treated with 0.5 to 1 μg of SH2 domain).

The immobilized SH2 domains herein, including the affinity enrichment steps, ELISA procedures, and binding competition assays may be produced in recombinant host cells, *e.g.*, bacteria, as fusion proteins using a protein which does not adversely affect expression and which enables the expressed fusion protein to be folded correctly. Herein, the SH2 domain was expressed as a bacterial fusion protein using either glutathione-S-transferase (GST) or maltose binding protein (MBP). Other experiments

were also conducted wherein SH2 domains from other proteins, v-src, c-src, and PLC (phospholipase C) were constructed. These experiments are depicted schematically in **Figure 1**.

Methods for designing vectors for the expression of fusion proteins, and their expression in recombinant host cells, *e.g.*, *E. coli* are well known in the art. *See*, *e.g.*, Sambrook *et al.*, MOLECULAR CLONING, 2nd Edition, which is incorporated by reference herein.

The particular scheme utilized for construction of MBP and GST fusion vectors for expression of SH2 domain containing fusion proteins is depicted in **Figure 2**. The Grb2 protein is commercially available as a GST fusion from Upstate Biotech Inc., Lake Placid, New York, USA. Antibodies to Grb2 and other SH2 domain containing proteins are also available from Upstate Biotech Inc. and from Santa Cruz Technology, Santa Cruz, California, USA. Vectors for directing the expression of GST and MBP fusion proteins are obtainable from Pharmacia Biotech Inc., Piscataway, New Jersey, USA and from New England Biolabs, Beverly, Massachusetts, USA, respectively.

The Grb2 SH2 protein can be produced in a variety of different forms and in a variety of host cells. One useful form of Grb2 SH2 is constructed by expressing the fusion protein as a soluble protein in baculovirus transformed host cells using standard methods. Another useful form comprises a signal peptide to facilitate protein secretion and for glycophospholipid membrane anchor attachment. This type of anchor attachment is referred to in the art as "PIG-tailing". *See*, Caras and Weddel, *Science*, **243**:1196-1198 (1989); and Lin *et al.*, *Science*, **249**:677-679 (1990), which are incorporated by reference herein. Using the PIG-tailing system, it is possible to cleave the receptor from the surface of SH2 expressing cells (*e.g.*, transformed CHO cells selected for high level expression of SH2 with a cell sorter) using phospholipase C. The cleaved SH2 protein still comprises a carboxy terminal sequence of amino acids, called the "HPAP tail," from the signal for membrane attachment and can be immobilized without further purification with an antibody specific for the HPAP tail (*e.g.*, the mAb 179).

The recombinant Grb2 SH2 fusion proteins can then be immobilized by coating the wells of micro-titer plates with an anti-HPAP tail antibody, blocking with bovine serum albumin (BSA) in PBS, and then binding the recombinant SH2 peptide to the antibody. Alternatively, the SH2-GSP fusion protein can be immobilized directly. The

immobilization reaction is preferably effected with various concentrations of Grb2-SH2 to determine the optimum amount for a given preparation. In addition, the immobilizing antibody is preferably completely blocked (with BSA or another suitable blocking compound) during the affinity enrichment process. Otherwise, unblocked antibody can potentially bind phage during the affinity enrichment procedure. For example, one can use peptides that bind to the immobilizing antibody to block any unbound sites, or simply immobilize the SH2 domain directly, without the use of an immobilizing antibody. *See* U.S. Patent Application Serial No. 947,339, filed September 18, 1992, which is incorporated by reference herein.

Peptides found to bind specifically to SH2 according to the described methods can then be synthesized as the free peptide and then tested in a phosphopeptide binding competition assay. This competition assay is conducted similarly to the ELISA, except that the density of the target GST-SH2 fusion protein in the wells is lower and a radiolabeled probe, *e.g.*, an autophosphorylated EGFR intracellular domain (ICD) or radio-iodinated phosphopeptide, is added in solution phase along with a peptide tested at various concentrations. Peptides that block the binding of the radiolabeled probe to SH2 domains are preferred compounds.

Using the above methods, a number of different fusion proteins were initially identified which bound to the tested SH2 domain. The DNA encoding the fusion proteins that bound to the receptor was then sequenced to determine the sequence of the random peptide contained in the fusion protein. The specific peptides identified in this initial screening are set forth in Table 1 below:

Table 1[1]

E C Y I N V P F T C M A GASAG

T E C Y L N V P E I C A GASAG

GGC D E V Y N N W S CGG

GGC L S Y M N S P M CGG

GGC Y E N L W P Y S CGG

GGC P E R Y E N V M CGG

[1] The amino acid sequences GASAG, CGG, GGGGS served as linker to join the rest of the peptide to the phage.

Q L Y E N W P V L T GGGGS
Q E R Y E N V P G I GGGGS
R E R Y E N V W Y V GGGGS

Analysis of these results indicates that a preferred SH2 binding peptide comprises the core sequence of amino acids $Z_1Z_2Z_3Z_4Z_5Z_6X$ where each X can be selected from any one of the 20 genetically coded L-amino acids, the stereoisomeric D-amino acids, or the non-naturally occurring amino acids; Z_1 is C, L, R, S, or V; Z_2 is tyrosine or an isostere thereof; Z_3 is E, I, L, M, or N; Z_4 is asparagine or an isostere thereof; Z_5 is L, S, V, or W; and Z_6 is M, P, S, or W, and wherein the tyrosine optionally can be phosphorylated. More preferably, the SH2 binding peptide comprises the core sequence $Z_7Z_1Z_2Z_3Z_4Z_5Z_6X$ where Z_7 is E, G, L, or Q, and Z_1-Z_6 and X are as set forth above. In a more preferred embodiment, the SH2 binding peptide comprises the core sequence $Z_7Z_1Z_2Z_3Z_4Z_5Z_6Z_8$ where Z_8 is C, E, F, G, M, P, V, or Y. Even more preferably, the SH2 binding peptide comprises the core sequence $Z_7Z_1Z_2Z_3Z_4Z_5Z_6Z_8$ where Z_1 is C or R; Z_3 is E or I; Z_5 is V; Z_6 is P or W; Z_7 is E; and Z_8 is C, E, F, G, M, P, V, or Y.

Particularly preferred SH2 binding peptides comprise the following core sequences: ECINVPFTCMA; TECYLNVPEICA; TECYLNVPEICA wherein the cysteines are joined by a disulfide bond; CDEVYVNWSC; CDEVYVNWSC wherein the cysteines are joined by a disulfide bond; CLSYMNSPMC; CLSYMNSPMC wherein the cysteines are joined by a disulfide bond; CYENLWpYSC; CYENLWpYSC wherein the cysteines are joined by a disulfide bond; CRERYENVMC; CRERYENVMC when the cysteines are joined by a disulfide bond; CPERYENVMC; CPERYENVMC when the cysteines are joined by a disulfide bond; QLYENWPVLT; QERYENVPGI; and RERYENVWYV.

The encouraging results from the initial panning efforts induced the inventors to continue and expand the RPD strategy to produce second and third generataion libraries.

2. SH2 Domain Selectivity

In addition to high affinity for the SH2 domain of Grb2, the goal of our screening and selection efforts is to identify leads that are specific for the particular target. As described above, this includes binding assays with radioligands that allow the quantification of this parameter of a lead compound. However, useful information can

also be obtained at a very early stage by testing, in a phage ELISA format, whether phage bearing peptides with affinity for the SH2 domain of Grb2 display detectable affinity for other SH2 domains. Such assays can be carried out using the above-described phage ELISA format that resulted in the original identification of the phage as Grb2-positive, but modified by including additional targets in the ELISA. (The results of such an assay are contained in **Figure 3**.) All phage tested in the indicated experiment appear to bear Grb2-specific peptides. This assay can be applied to phage expressing peptides that result from panning on other SH2 domain targets. Moreover, this experiment can be repeated with other SH2 domain targets in order to further determine the binding selectivity of the peptides. SH2 domains are well known in the art and can be made, *e.g.*, by peptide synthesis procedures and recombinant methods.

3. First Generation Peptides

Continued panning of the original RPD libraries (a random 10mer and a random 8mer in a disulfide backbone (CX_8C) expressed on the pVIII protein) revealed many additional Grb2-specific phage. The deduced peptide sequences from several are shown in Table 2.

Table 2[2]

C8C library	10mer library
GGC L S Y M N S P M CEG	R S G Y E N W P V I GGGDS
GGY D E L Y E N W P CGG	G D E H Y R N S L GGGGS
GGC D E V Y V N W S CGG	E D E R Y M N L P W GGGGS
GGC M E E Y V N W S CGG	E E R Y M N V M P F GGGGS
GGC Q E E Y V N W S CGG	S S E R Y E N V I F GGGGS
GGC V H Y E N Y M W CGG	E E Q Y V N M P W F GGGGS
GGC Y V N V Y D P L CGG	Q R E K Y E N W P F GGGGS
GGC Y W Q N V P E S CGG	

4. Second Generation Peptides and Affinity Discrimination

Because of the positive results obtained from the original panning experiments using Grb2 SH2 as target, new libraries were designed with the goal being the identification of even higher affinity leads. The first custom library contained the

[2] Peptide sequences from panning Grb2 SH2 with the indicated pVIII RPD libraries.

sequence X_5YXNX_8, expressed on the amino terminus of pVIII. This library was screened as described *supra* for five rounds and also under new conditions using the peptide AF-10375 (FLPVEPYpYINQSVP-NH_2), during washing (after rounds 2 through 5), in order to enrich for phage bearing peptides of higher affinity. This "peptide elution" strategy has resulted in this desired goal. Individual phage from the second round (after 1 round of "normal" elution and 1 round of peptide elution (or normal elution as a control)) were assayed by phage ELISA. Positives were identified in both cases (*see* **Figure 4**). The peptides encoded by these clones are presented in Table 3.

Table 3[3]

I S D E C Y V N V W D S H D L C GGGGS	T P E L D Y V N W K S G A A Y C GGG
T S S E R Y V N D V V W L Y S S GGGG	Q R E W H Y E N W S E I G P C C GGGGS
G F D E C Y V N I L D S W C V M GGS	K C Y Q E Y V N L P C L P X GGX
D M C E Q Y Q N W T A P C I L E GGGGS	G L C E Q Y V N L I G P C G H Y GGGGS
G G S C G Y V N Y C E L E N R L GGGGS	N H E S N Y E N W P P S T L T T GGGGS

5. Alternative SH2 Domains

Additional panning has been carried out with a variety of libraries on several targets, including the SH2 domains of src, Shc, and the C-terminal domain of PLCγ1. These efforts are aimed at identifying sequences that can serve as probes for the development of the binding assays to measure specificity (as described *supra*). A hit was obtained from Shc and its sequence was

RPD-derived peptide	E G C S S E P Y G V W C GGGGS

6. Analog Preparation

A series of compounds was synthesized based on the cyclic peptide (E C pY I N V P F T C), a phosphorylated 10mer with a 9-membered disulfide-bridged loop, that had an IC_{50} of about 0.03 μM. To investigate the effect of ring size on affinity, the second Cys was shifted to generate derivatives with 7- and 6-membered rings (*see* end of Table 4 below).

One example of the effects of cyclization on affinity is particularly noteworthy. An RPD-derived linear peptide was cyclized by the addition of flanking Cys residues (*see*

[3] Peptide sequences from panning Grb2 SH2 with the custom X_5-Y-X-N-X_8 library.

end of Table 4). This cyclization had no effect on the unphosphorylated peptide (compare (R E R Y E N V P G -NH_2), ~3.2 μM and (C R E R Y E N V W Y C -NH_2), 4 μM), but resulted in a remarkable (22-fold) increase in potency for the phosphorylated peptide (compare (R E R pY E N V P G -NH_2), ~0.42 μM and (C R E R pY E N V W Y C -NH_2), 0.008 μM).

Table 4

sequence	IC_{50} (μM)
F L P V P E pY I N Q S V P -NH_2	+
F L P V P E Y I N Q S V P -NH_2	-
Ac- C pY I N V P F T C -NH_2	++
Ac- C pY I N V P F T C -NH_2	++
E C pY I N V P F T C	++
E C pY I N V P F T C	++
E C Y I N V P F T C	+
E C Y I N V P F T C	+
Ac- C D E V pY V N W S C -NH_2	++
Ac- C D E V pY V N W S C -NH_2	++
C D E V pY V N W S C -NH_2	++
C D E V pY V N W S C -NH_2	++
Q E R pY E N V P G -NH_2	+
Q E R Y E N V P G -NH_2	-
R E R pY E N V P G -NH_2	++
R E R Y E N V P G -NH_2	+
E C pY I N V P F T C -NH_2	++
E C pY I N V P C T -NH_2	++
E C pY I N V C F T -NH_2	+
C R E R pY E N V W Y C -NH_2	++
C R E R Y E N V W Y C -NH_2	+

Table 4. The indicated peptides, derived from RPD, were synthesized, and the structures were verified by HPLC and MS. In this competition assay, the probe was autophosphorylated EGFR-ICD. Linear peptides were assayed in buffer containing 10 mM DTT; this treatment did not affect binding of the reference peptide FLPVPEpYINQSVP-NH_2. Underlining indicates cyclization via a disulfide bond.

B. Identification of Core Peptides

1. ESL Methods

a. libraries based on EGFR-ICD

In an effort to identify the optimal substitution pattern around the tyrosine/phosphotyrosine residue, a collection (or library) of peptides bound to beads was constructed. *See* co-pending U.S. Patent Application Serial No. 07/946,239, filed September 16, 1992 and PCT Patent Publication No. 92/00091, both of which are incorporated herein by reference. The library was based on the sequence surrounding the tyrosine residue Y1068 of the epidermal growth factor receptor intracellular domain (EGRF-ICD) and consisted of bead bound-peptides having the sequence LPX_{-3}-X_{-2}-X_{-1} pYINQSV.[4] All natural amino acids except cysteine and tryptophan were utilized at each variable position, and the phosphotyrosine residue is indicated by pY. The total degeneracy was $18^3=5382$.

The techniques for selection of individual beads displaying SH2-binding ligands on their surface are analogous to fluorescence activated cell sorting (FACS) methods for cloning mammalian cells expressing cell surface antigens or receptors. Therefore, methods for selecting and sorting beads will be readily apparent to those skilled in the art of cell sorting. For example, an SH2 domain can be labeled with a fluorescent tag and then incubated with the mixture of beads displaying oligomers. After washing away unbound or non-specifically bound domains, one can then use FACS to sort the beads and to identify and isolate physically individual beads showing high fluorescence.

Identification of the positive SH2-binding phosphotyrosine peptides in the peptides-on-beads libraries described herein utilized an antibody staining assay employing the three different staining reagents: the SH2-GST fusion protein, rabbit anti-GST IgG, and phycoerythrin (PE) - conjugated goat anti-rabbit. A schematic representation of the assay for a peptide attached to a bead is shown in **Figure 5**. The Grb2-GST fusion protein

[4] Residues to the right of the tyrosine/phosphotyrosine (i.e., closer to the carboxy terminus) are numbered sequentially with the tyrosine/phosphotyrosine being zero and the immediately adjacent residue being +1. Residues to the left of the tyrosine/phosphotyrosine (i.e., closer to the amino terminus) are numbered sequentially with the tyrosine/phosphotyrosine being zero and the immediately adjacent residue being-1.

binds the peptide, and the entire complex is bound by rabbit anti-GST IgG, followed by phycoerythrin-conjugated goat anti-rabbit immunoglobulin.

Alternatively, the detection assay shown in **Figure 6** may be employed. In this assay, the peptide is recognized by an MBP Grb2-179 fusion protein, which contains the HPAP epitope. The protein may then be detected by conventional detection means, for example, by the use of a Cy3 (reactive cyanamine fluorescence-based detection, Biologial Detection Systems Inc., Pittsburgh, PA) labelled Ab179 IgG (which recognizes the HPAP epitope referred to above).

The entire library of 11-mers having the sequence L-P- X_3-X_2-X_1-pY-I-N-Q-S-V-bead tested positive under the assay conditions shown in **Figure 5**.

b. preferred core peptide sequences

To further define the ability of the region adjacent to the phosphotyrosine to accommodate substitutions, an encoded phosphotyrosine peptide library having the structure A pY - X_1 -X_2 -X_3 -S - V -bead - oligotag was constructed using the encoded synthetic library technology described in co-pending U.S. Patent Application Serial No. 07/946,239, filed September 16, 1992. *See* also PCT Patent Publication No. 92/00091, incorporated herein by reference. Residues X_1, X_2, and X_3 were taken from a set of 26 building blocks including 19 natural L-amino acids (except cysteine), the D-isostereomers of Gln, Asn, and Ile, and the unnatural amino acids cyclohexylalanine (Cha), methionine sulfoxide (Mso), norvaline (Nva), and norleucine. The library included 17,576 (26^3) different peptides.

The ratio of peptide to oligonucleotide tag on each bead was approximately 1000:1. Twenty-six different 3-base codons were employed using the bases 7-deaza-A, C, and T. Standard acid-labile amino acid side chain protecting groups were employed, and the phosphate group in phosphotyrosine was not protected during library construction. Positive and negative control parallel synthesis beads comprised beads coated with an oligonucleotide and either EpYINQSV or the non-phosphorylated analog of this sequence, EYINQSV.

Following the sorting of individual beads exhibiting high fluorescence (when treated with the detection reagents described in **Figure 5**), the oligonucleotides on the beads were amplified by PCR and the soluble DNA was then sequenced. Representative X_1, X_2, and X_3 sequences from the gate 1 beads, which demonstrated the highest

fluorescence, are shown in **Figure 7**. The population in gate 1 represented 0.1% of the library population. Representative peptides from gate 1 were synthesized, and IC_{50}'s, determined in the radiolabelled phosphopeptide competition assay, are shown in parentheses.

Analysis of these results indicates that a preferred SH2 binding peptide comprises the core sequence $Z_9XZ_{10}XSV$, where each X can be independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; Z_9 is phosphotyrosine or an isostere thereof; and Z_{10} is asparagine or an isostere thereof. Preferably, the SH2 binding peptide comprises the core sequence $Z_9Z_{13}Z_{10}XSV$ where Z_{13} is A, E, I, L, M, N, or norvaline. Particularly preferred SH2 binding peptides include: ApYLNKSV; ApYENKSV; ApYLNESV; ApYNvaNASV; ApYANFSV; and ApYNvaNGSV.

c. phosphotyrosine walk libraries

To further ascertain the effect of substitution around the phosphotyrosine on the binding of the core peptide and whether the "INQSV" region (i.e., the region adjacent to the phosphotyrosine) could be optimized, a phosphotyrosine "walk" library of 6-mers was prepared using the peptides-on-beads system. In this study, the phosphotyrosine group was systematically shifted from the N- to the C-terminal of the peptide. Alanine residues were introduced at either end of the peptide to retain the natural, peptide-like environment.

Thus, four phosphopeptide libraries were prepared:

(1) A - pY - X_1 -X_2 -X_3 - A - Bead;

(2) A - X_{-1} - pY - X_1 -X_2 - A - Bead;

(3) A - X_{-2} - X_{-1} - pY - X_1 - A - Bead;

(4) A - X_{-3} - X_{-2} - X_{-1} - pY - A - Bead;

The first library was assayed and found to contain several peptides capable of binding to Grb2. After FACS sorting and microsequencing of the brightest 1% of beads, it was discovered that asparagine was strongly preferred at the X_2 position, while the X_1 and X_3 positions could accommodate a wide variety of amino acids. The X_1 position showed a slight enrichment of the following amino acids: G, Y, M, V, F, I and L, whereas the X_3 showed a slight enrichment of the following amino acids: Q, E, M, V, F, I, and L. Peptides showing binding to SH2 domains were also identified in the second

library. However, the latter two libraries possessed no peptides showing detectable activity against Grb2. These results provide further evidence that asparagine at position X_2 provides for good SH2 domain binding. These libraries, given their generic nature, should result in the identification of other positive hits (SH2 domain binding peptides) using Grb2 or other SH2 domains as the target.

d. D-amino acid scan libraries

Further libraries were prepared to explore the ability of the phosphopeptides to tolerate substitution with D-amino acids or alanine. For the D-amino acid scan library, the X_2 position (i.e., the position two residues from the phosphotyrosine) was maintained as asparagine, while the more tolerant X_1 and X_3 positions were substituted with 18 D-amino acids, excluding cysteine and tryptophan. The D-amino acids were substituted either individually or simultaneously to provide the following libraries:

A - pY - X_1 - Asn - dX_3 - A - bead;

A - pY - dX_1 - Asn - X_3 - A - bead; and

A - pY - dX_1 - Asn - dX_3 - A - bead.

Again, alanine residues were introduced at each end of the peptides to provide an enhanced peptide-like environment around the molecules. Each library has a degeneracy of $18^2=324$.

The libraries were analyzed by antibody staining and FACS as described above to further characterize optimization of peptide sequences. 36% of the beads of the first library yielded positive results under the assay, indicating that D-amino acids may be tolerated at the X_3 position. In addition, D-amino acids could be tolerated marginally at the X_1 positions, as indicated by 0.3% of the beads of the second library yielding positive results.

e. general uses of ESL libraries

As discussed previously, SH2 domains are found in a diverse collection of proteins. Moreover, the amino acid residues immediately surrounding the phosphotyrosine residue of the ligand is known to be critical to the binding of the ligand. Thus, although the phosphotyrosine walk, D-amino acid scan, and alanine scan libraries have been exemplified in conjunction with the SH2 domain of Grb2, one of skill of the art will appreciate that these libraries will also find use in the identification of ligands capable of specifically binding SH2 domains of other proteins, such as the src family,

abl, syk, PTP1C, PLC ., GAP, SHPTP2, Vav, p85, p13K, c-Crk, SHC, Nck, ISGF3, and Sem-5.

These libraries will comprise a plurality of members, typically at least 32, preferably between about 32 and 1,000,000, and more preferably between about 5,000 and 10,000. Each member will comprise a solid support which has been coupled to a peptide sequence and optionally, a tag sequence. For the phosphotyrosine walk library, the peptide sequence will comprise a sequence of amino acids selected from the group consisting of:

pY - X - X - X - X;
X - pY - X - X - X;
X - X - pY - X - X; and
X - X - X - pY- X;

where pY is phosphotyrosine; each X can be selected any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acid.

For the alanine scan library, the peptide will comprise a sequence of amino acids selected from the group consisting of:

pY - A - X - X - X;
pY - X - A - X - X; and
pY - X - X - A - X;

where pY is phosphotyrosine; each X can be selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids.

For the D-amino scan library, the peptide will comprise a sequence of amino acids selected from the group consisting of:

pY - dX - X - X - X;
pY - X - dX - X - X;
pY - X - X - dX - X; and
pY - dX - X - dX - X;

where pY is phosphotyrosine; each X can be selected from any one of the 20 genetically coded-L-amino acids; each dX can be selected from any one of the 20 D-amino acids which are stereoisomeric to the 20 genetically coded-L-amino acids.

Each of the libraries will contain at least one member capable of binding to an SH2 domain with a binding affinity of less than about 1 x 10^{-4} M, and preferably less

than about 1 x 10^{-5}. Although in some instances, it will be desirable to have SH2 binding peptide capable of binding to more than one type of SH2 domain, preferably, the binding of this member will be specific for a single SH2 domain of interest.

f. minimum peptide length

For the design of libraries of peptides-on-beads, it is desirable to use the minimum framework that result in the selection of high affinity compounds. Competition assays with soluble peptides (*see* later) are aimed at determination of the minimum pharmacophore. To complement those efforts and determine their relevance to assays of peptides immobilized on beads, we assayed (on untagged beads) peptides with the sequences listed in Table 5. These peptides were synthesized on the beads. Final Fmoc numbers were very similar, indicating roughly comparable synthetic efficiencies and, therefore, peptide densities on the beads. The data from this experiment suggest that the minimum peptide length is 4 or 5 residues, as long as the amino terminus is acetylated. C-terminal extension by 2 additional residues improves the fluorescence more than the affinity (*see* Table 5). Whether this improvement is caused by the amino acids themselves or is a function of increased distance from the surface of the bead has not been established.

Table 5[5]

Sequence	IC_{50}	Fluoresc.	Sequence	IC_{50}	Fluoresc.
pYINQ	28	50	Ac-pYINQSV	n.d.	1785
Ac-pYINQ	20	1505	EpYINQSV	+	n.d.
EpYINQ	11	720	Ac-EpYINQSV	+	n.d.
Ac-EpYINQ	11	1430	VPEpYINQSV	+	2500

Affinity Discrimination

ESL technology also allows affinity discrimination among a population of beads. Several different populations of beads are used which present unique peptides of known affinity in the competition assay (*see* Table 6). These sequences were derived originally

[5] Dependence of fluorescence intensity on peptide sequence. Untagged beads stained with Grb2 SH2 and analyzed by FACS. Median fluorescence intensity of the populations is indicated. For blank beads, the fluorescence was 4. IC_{50}'s were determined with the iodo-phosphopeptide probe and "nd" means not determined.

from the ESL experiments described *supra*. These peptides were synthesized on the beads, at comparable yields. The beads were stained and washed and then allowed dissociation of the staining reagents. When this task was accomplished in the presence of 100 μM (F L P V P E pY I N Q S V P -NH_2) a 13mer phospho-peptide with a 1 μM IC_{50}, we captured the released SH2 and prevented rebinding to peptide on the beads. **Figure 8** shows a FACS analysis of peptides-on-beads. Addition of peptide during the dissociation caused a rapid loss of fluorescence for both "high" and "low" affinity peptides.

Table 6

name	sequence	IC_{50}
MN1	A*YdQDSV	-
MN2	A*YENKSV	+
MN3	A*YLNESV	+
MN4	A*YHNKSV	-

Table 6. Peptides derived from ESL and synthesized as control populations.

Other experiments were conducted wherein the conditions of the dissociation were varied in order to define conditions that would give differentiation between the low and high affinity peptides. In the experiments shown in **Figure 9**, beads were incubated with the SH2 reagents, washed, and allowed to incubate overnight at room temperature, in buffered saline without or with the addition of the reference peptide (F L P V P E pY I N Q S V P -NH_2) at 100 μM. In the absence of peptide, dissociation resulted in substantial (though incomplete) loss of fluorescence on samples analyzed. With peptide addition, the signal due to the lowest affinity peptides dropped down to background levels, while the higher affinity peptides retained some signal. Thus, this assay was able to discriminate affinity in a population of beads. This assay can be further refined by one of ordinary skill, *e.g.*, by the use of other peptides having known affinity, use of alternative staining reagents by variation of dissociation conditions, etc., to ascertain optimal conditions for affinity discrimination among a population of beads.

Inspection of the values of fluorescence intensity for beads bearing peptides of different affinities further reveals that higher affinity peptides result in significantly higher fluorescence. Conversely, beads with the lower affinity peptides exhibit lower

fluorescence. (This difference is not due to different synthetic efficiencies.) As discussed *supra*, the reagent cocktail for detecting binding of an SH2 domain to an immobilized phosphopeptide is a double sandwich of Cy3-labeled-antibody 179 and MBP-SH2-179. Cy3 is a small-molecule fluorophore with spectral properties similar to rhodamine. This two-step combination, wherein the complex is formed prior to incubation with the ESL beads, appears to be the favored protocol.

C. Peptidomimetics

1. Overview

In addition to peptides consisting only of naturally-occurring amino acids, peptidomimetics or peptide analogs are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, *J. Adv. Drug Res.*, **15**:29 (1986); Veber and Freidinger, TINS, p.392 (1985); and Evans *et al.*, *J. Med. Chem.*, **30**:1229 (1987), which are incorporated herein by reference). Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent or enhanced therapeutic or prophylactic effect. Moreover, such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad spectrum of biological activities), reduced antigenicity, and others.

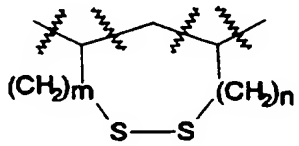
2. Cyclic Analogs

As discussed previously, the invention embraces SH2 binding peptides which contain two or more residues which permit cyclization to occur. As discussed, it has been discovered that cyclized SH2 binding peptides, preferably those which are also phosphorylated (i.e., certain phosphotyrosine), can have desirable properties, i.e., enhanced inhibitory activity.

Methods for cyclization of peptides are well known in the art, as are residues which permit cyclization to occur. (*See*, *e.g.*, *J. Pharm. Sci.*, Vol. 61 (9), 1345-1356 (1972); Hruby *et al.*, *Life Sciences*, **31**:189-199 (1982) incorporated by reference herein.) The invention is not restricted to any specific means of cyclization, or any specific residues to facilitate cyclization. Residues which facilitate cyclization include by way of

example cysteine, homocysteine, penicillamine, β-mercapto-β,β-cyclotetra-methylpropionic acid (Pmp) and derivatives thereof. The invention further embraces heterodetic and homodetic cyclized compounds. Preferably, the residues which allow for cyclization will be introduced at the N- and C-terminal end of a SH2 peptide to produce cyclic structures ranging in size from about 3 to 12 residues. Based on the observed results, it appears that cyclic structures ranging in size from about 6 to 11 residues, and more preferably ranging in size from about 9 to 11 residues exhibit desirable SH2 inhibitory properties. It further appears that these cyclic peptides, in order to obtain optimal inhibitory results, should further be phosphorylated.

When cysteine or homocysteine are selected as the cyclization effecting residues, the cyclized form which comprises an intramolecular disulfide bond can be represented schematically below:

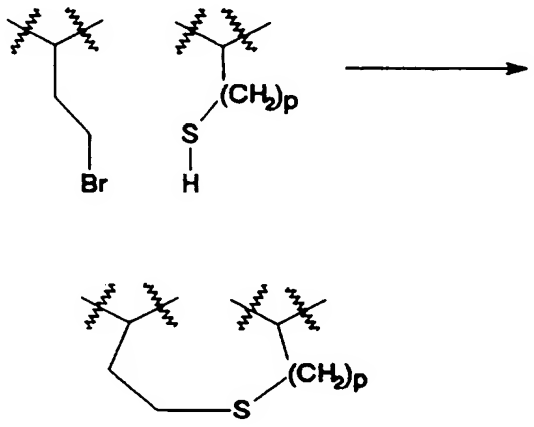


wherein m and n are independently 1 or 2 (2 for homocysteine).

One of skill of the art will readily appreciate that a cyclic structure can be introduced at various locations along the peptide backbone, i.e., the cyclic structure can be formed from the entire peptide sequence or fragments thereof. For example, cysteines (or other residues capable of intramolecular cyclization) can be introduced within a peptide backbone, at positions adjacent to the sequence -X-p-Y-X- and cyclized to yield a cyclic peptide containing a phosphotyrosine and having two peptidic appendages (i.e., the remaining portions of the original peptide sequence). Alternatively, if cysteines (or other residues capable of intramolecular cyclization) are introduced at positions adjacent to the sequence -X-N-X, the asparagine residue will reside within a cyclic framework. Finally, cysteines (or other residues capable of intramolecular cyclization) can be introduced at

both termini of the peptide. Formation of the disulfide bond will then transform the linear peptide, in its entirety, to a cyclic peptide.

Other embodiments of this invention provide for analogs of these disulfide derivatives in which one of the sulfurs has been replaced by a CH_2 group or other isostere for sulfur. These analogs can be prepared from the compounds of the present invention having at least two C or Hoc residue, wherein one of the C or Hoc residues has been replaced with a α-amino-γ-bromobutyric acid, via an intramolecular or intermolecular displacement, using methods known in the art as shown below:



wherein p is 1 or 2. One of skill in the art will readily appreciate that this displacement can also occur using other homologs of α-amino-γ-bromobutyric acid and/or homocysteine.

Alternatively, the amino-terminus of the peptide can be capped with an alpha-substituted acetic acid, wherein the alpha substituent is a leaving group, such as an α-haloacetic acid, for example, α-chloroacetic acid, α-bromoacetic acid, or α-iodoacetic acid. The compounds of the present invention having a C or Hoc residue can be cyclized via displacement of the leaving group by the sulfur of the C or Hoc residue. *See, e.g.*, Barker *et al., J. Med. Chem.*, **35**:2050-2048 (1992); and Or *et al., J. Org. Chem.*, **56**:3146-3149, each of which is incorporated herein by reference.

Particularly preferred cyclic SH2 binding peptides of the present invention include: CpYINQC; CpYCNQ; CEpYCNQ; pYCNC; EpYCNC; CpYINVPFTC; TECpYLNVPEICA; CDEVpYNNWSC; CDEVpYVNWSC; CLSpYMNSPMC;

CpYENLWPYSC; CRERpYENVMC and CPERpYENVMC, wherein the cysteines are joined by a disulfide bond. Moreover, it is expected that the cyclization peptides listed in Table 4 will result in SH2 peptides having desirable properties as well.

As discussed previously, it can be seen that the preferred cyclic peptides contain phosphotyrosine.

3. Non-Naturally Occurring Amino Acids

These procedures can also be used to synthesize peptides in which amino acids other than the 20 naturally occurring, genetically encoded amino acids are substituted at one, two, or more positions of any of the compounds of the invention. For instance, naphthylalanine can be substituted for tryptophan, facilitating synthesis. Other synthetic amino acids that can be substituted into the peptides of the present invention include L-hydroxypropyl, L-3,4-dihydroxyphenylalanyl, δ amino acids such as L-δ-hydroxylysyl and D-δ-methylalanyl, L-α-methylalanyl, β amino acids, and isoquinolyl. D amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides of the present invention.

One can replace the naturally occurring side chains of the 20 genetically encoded amino acids (or D amino acids) with other side chains, for instance with groups such as alkyl, lower alkyl, cyclic 4-, 5-, 6-, to 7-membered alkyl, amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, carboxy and the lower ester derivatives thereof, and with 4-, 5-, 6-, to 7-membered heterocyclic. In particular, proline analogs in which the ring size of the proline residue is changed from 5 members to 4, 6 or 7 members can be employed. Cyclic groups can be saturated or unsaturated, and if unsaturated, can be aromatic or non-aromatic.

4. Phosphotyrosine Isosteres

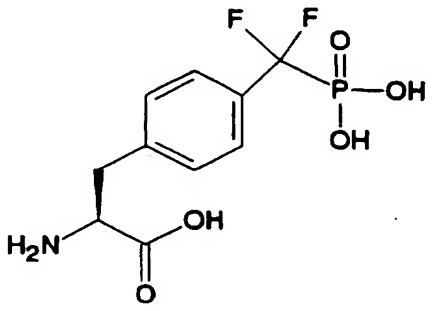
In some embodiments of the invention, the peptides contain phosphotyrosine residues. In some applications, the phosphotyrosines may be generated by global phosphorylation of the peptide libraries. However, this post-synthesis phosphorylation may be incompatible with amino acid residues bearing oxidizable side chains such as tryptophan and methionine. In addition, phosphotyrosine is hydrolytically unstable. Thus, in a preferred embodiment of the invention, the phosphotyrosine may be replaced by a tyrosine or phosphotyrosine derivative, analog or isostere.

Structures of suitable tyrosine and phosphotyrosine replacements are provided in **Figure 10** and include dimethyl phosphotyrosine, "methylene phosphonic" analogs and "ether phosphonic" analogs, "difluorophosphonic" analogs, "ether carboxylic" analogs, "constrained" analogs.

Specific examples of phosphotyrosine and tyrosine analogs isosteres include those described in WO 94/11392, by Dobrusin, published on May 26, 1994; WO 94/07913 by Dobrusin *et al.*, published on April 14, 1994; Burke *et al.*, *Biochemistry*, **33**:6490-6494 (1994); Burke *et al.*, *J. Med. Chem.*, **34**:1577-1581 (1991) which specific compounds and methods of synthesis are incorporated by reference herein. Specific examples of preferred tyrosine and phosphotyrosine analogs and isosteres include by way of example tyr($CH_2PO_3H_2$), tyr(SO_3H), tyr(CH_2CO_2tBu), tyr(CH_2CO_2OH), tyr($CH_2PO_3Et_2$), 4-phosphono(difluoromethyl)-L-phenylalanine (F_2Pmp), N-Fmoc α-Me O-phosphotyrosine, AcF_2Pmp, Fmoc-D-tyr(PO_3H_2)OH, Fmoc-(α-Me) tyr(PO_3H_2)-OH, Fmoc-L-Tyr(CH_2CO_2tBu)-OH, Ac-L-Phe($CH_2PO_3Bu_2$)-OH, N^{α}-Fmoc-L-Tyr($CH_2PO_3H_2$)-OH, N^{α}-Cbz-Tyr($CH_2PO_3Et_2$)-OH, N^{α}-Cbz-L-Tyr($CH_2PO_3Et_2$)-OMe, L-Tyr($CH_2PO_3Et_2$)-OH, N^{α}-Fmoc-L-Tyr($CH_2PO_3Et_2$)-OH, N^{α}-Fmoc-L-Tyr($CH_2PO_3H_2$)-OH, and MeNpoc-L-Tyr[PO_3H_2]-OH.

These tyrosine and phosphotyrosine analogs can be substituted for tyrosine or phosphotyrosine in the SH2 binding peptides of the invention or those known in the art.

Particularly preferred isosteres and/or analogs include



and the methyl and dimethyl derivatives thereof. The preparation of phosphotyrosine and tyrosine analogs and tyrosine isosteres is disclosed *infra* in the examples and in the references incorporated by reference. These specific isosteres and analogs only represent a few of those which may be used in the present invention.

It should be noted that these isosteres and analogs may be incorporated into the peptides on beads, whether tagged or untagged, as required.

5. Asparagine Isosteres

In other embodiments of the invention, the asparagine residue may be replaced by an asparagine derivative, analog, or isostere, which may be incorporated into the peptides on beads, whether tagged or untagged, as required. Structures of suitable asparagine replacements are provided in **Figure 11**.

Particularly preferred isosteres and/or analogs include Fmoc-L-Asp[3-(1,3-dithian-2-yl)]-OH, Fmoc-L-Asp[3-NHCHO]-OH, Fmoc-L-Asp[3-SO_2NHTrt]-OH, Ala[3-NHAc], Ala[3-NHCHO], Fmoc-Ala[3-NHAc]-OH, Fmoc-Ala[3-NHCHO]-OH, Fmoc-Ala[SO_2NHTrt]-OH, Fmoc-Ala[3-SO_2NH-Het]-OH where Het equals a purine base or another pharmacophore. It should be noted that these isosteres and analogs may be incorporated into the peptides on beads, whether tagged or untagged, as required.

6. Backbone Replacements

The peptide compounds of the invention can also serve as structural models for non-peptidic compounds with similar biological activity. *See* Hruby *et al.*, *Biochem. J.*, **268(2):**249-262 (1990), incorporated herein by reference. Those of skill in the art recognize that a variety of techniques are available for constructing compounds with the same or similar desired biological activity as the lead peptide compound but with more favorable activity than the lead compound with respect to solubility, stability, and susceptibility to hydrolysis and proteolysis. *See* Morgan and Gainor, *Ann. Rep. Med. Chem.*, **24:**243-252 (1989), incorporated herein by reference.

Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as naturally-occurring receptor-binding polypeptide, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH_2NH-, CH_2S-, CH_2-CH_2, -CH=CH- (*cis* and *trans*), -$COCH_2$-, -CH(OH)CH_2-, and CH_2SO-, by methods known in the art and further described in the following references: Spatola, A.F., CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES, AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A.F., VEGA DATA (March 1983), Vol. 1, Issue 3, *Trends Pharm. Sci.*, 463-468 (general review) (1980); Hudson, D. *et al.*, *Int. J. Pept. Prot. Res.*, **14:**177-185 (-CH_2NH-,CH_2CH_2-) (1979); Spatola *et al.*, *Life Sci.*,

38:1243-1249 (-CH_2-S) (1986); Hann *J. Chem. Soc. Perkin Trans. I*, 307-314 (-CH-CH-, *cis* and *trans*) (1982); Almquist *et al., J. Med. Chem.*, **23**:1392-1398 (-$COCH_2$-) (1980); Szelke *et al., European Appln. EP 45665* **CA**:97:39405 (-$CH(OH)CH_2$-) (1982); Holladay *et al., Tetrahedron Lett.*, **24**:4401-4404 (-$C(OH)CH_2$-) (1983); and Hruby *Life Sci.*, **31**:189-199 (-CH_2-S-) (1982); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH_2NH-.

D. Phosphorylated Peptides

As can be seen from the above discussion, the phosphorylated SH2 binding peptides comprise a core sequence of amino acids $Z_9XZ_{10}X$, where each amino acid is indicated by standard one letter abbreviation, each X can be selected from any one of the 20 genetically coded L-amino acids, the stereoisomeric D-amino acids and non-naturally occurring amino acids; Z_9 is phosphotyrosine or an isostere thereof; Z_{10} is asparagine or an isostere thereof; and the amino terminus is acylated, provided that if Z_9 is phosphotyrosine, Z_{10} is asparagine, then the peptide is not $GDGZ_7XZ_8XSPLLL$.

Preferably, the SH2 binding peptide will comprise the core sequence $XZ_9XZ_{10}X$ where each X can be independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids. More preferably, the SH2 binding peptide will comprise the core sequence $Z_{11}Z_9XZ_{10}X$ where Z_{11} is C, E, L, R, S or V. In a more preferred embodiment, the SH2 binding peptide will comprise the core sequence $Z_9XZ_{10}XZ_{12}$ where Z_{10} is C, M, P, S or W, and more preferably, the core sequence $Z_{11}Z_9XZ_{10}XZ_{12}$ where Z_{11} is C, E, L, R, S or V; and Z_{12} is C, M, P, S or W; and wherein if both Z_{11} and Z_{12} are C, then the cysteines optionally are joined by a disulfide bond. In a yet more preferred embodiment, the SH2 binding peptide will comprise the core sequence $Z_{11}Z_9Z_{13}Z_{10}XZ_{12}$ where Z_{13} is E, I, L, M or N. Most preferably, the SH2 binding peptide will comprise the core sequence $Z_{11}Z_9Z_{13}Z_{10}Z_{14}Z_{12}$ where Z_{14} is L, Q, S, V or W.

Particularly preferred phosphorylated SH2 binding peptides include EpYINQ; pYINQ; pYANA; EpYANA; CpYINQC; CpYINQC wherein the cysteines are joined by a disulfide bond; CpYCNQ; CpYCNQ wherein the cysteines are joined by a disulfide bond; CEpYCNQ wherein the cysteines are joined by a disulfide bond; pYCNC wherein the cysteines are joined by a disulfide bond; EpYCNC; EpYCNC wherein the cysteines are joined by a disulfide bond; CpYINVPFTC; CpYINVPFTC wherein the cysteines are

joined by a disulfide bond; FLPVPEpYINQSVP; ECpINVPFTCM; TECpYLNVPEICA; TECpYLNVPEICA wherein the cysteines are joined by a disulfide bond; CDEVpYVNWSC; CDEVpYVNWSC wherein the cysteines are joined by a disulfide bond; CLSpYMNSPMC; CLSpYMNSPMC wherein the cysteines are joined by a disulfide bond; CpYENLWPYSC; CpYENLWPYSC wherein the cysteines are joined by a disulfide bond; CPERpYENVMC; CPERpYENVMC wherein the cysteines are joined by a disulfide bond; CRERpYENVMC; CRERpYENVMC wherein the cysteines are joined by a disulfide bond; QLpYENWPVLT; QERpYENVPGI; and RERYENVWYV.

1. Synthesis

As noted above, the peptides of the invention may be prepared by classical methods known in the art by using standard solid phase techniques. The standard methods include exclusive solid phase synthesis, partial solid phase synthesis, fragment condensation, classical solution synthesis, and recombinant DNA technology. *See, e.g.*, Merrifield, *J. Am. Chem. Soc.*, **85:**2149 (1963) incorporated herein by reference. On solid phase, the synthesis is typically commenced from the C-terminal end of the peptide using an α-amino protected resin. A suitable starting material can be prepared, for instance, by attaching the required α-amino acid to a chloromethylated resin, a hydroxymethyl resin, or a benzhydrylamine resin. One such chloromethylated resin is sold under the trade name BIO-BEADS SX-1 by Bio Rad Laboratories, Richmond, CA, with the preparation of a hydroxymethyl resin being described by Bodonszky *et al.*, *Chem. Ind.* (London) **38:**1597 (1966). The benzhydrylamine (BHA) resin has been described by Pietta and Marshall, Chem. Comm. 650 (1970) and is commercially available from Beckman Instruments, Inc., Palo Alto, CA, in the hydrochloride form.

Thus, the compounds of the invention can be prepared by coupling an α-amino protected amino acid to the chloromethylated resin with the aid of, for example, cesium bicarbonate catalyst, according to the method described by Gisin, *Helv. Chim. Acta*, **56:**1467 (1973). After the initial coupling, the α-amino protecting group is removed by a choice of reagents including trifluoroacetic acid (TFA) or hydrochloric acid (HCl) solutions in organic solvents at room temperature.

The alpha-amino protecting groups are those known to be useful in the art of stepwise synthesis of peptides. Included are acyl type protecting groups (*e.g.*, formyl, trifluoroacetyl, acetyl), aromatic urethane type protecting groups (*e.g.*, benzyloxycarboyl

(Cbz) and substituted (Cbz), aliphatic urethane protecting groups (*e.g.*, t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl) and alkyl type protecting groups (*e.g.*, benzyl, tr-phenylmethyl). Boc and Fmoc are preferred protecting groups. The side chain protecting group remains intact during coupling and is not split off during the deprotection of the amino-terminus protecting group or during coupling. The side chain protecting group must be removable upon the completion of the synthesis of the final peptide and under reaction conditions that will not alter the target peptide.

The side chain protecting groups for Tyr include tetrahydropryranyl, tert-butyl, trityl, benzyl, Cbz, Z-Br-Cbz, and 2,5-dichlorobenzyl. The side chain protecting groups for Asp include benzyl, 2,6-dichlorobenzyl, methyl, ethyl, and cyclohexyl. The side chain protecting groups for Thr and Ser include acetyl, benzoyl, trityl, tetrahydropyranyl, benzyl, 2,6-dichlorobenzyl, and Cbz. The side chain protecting groups for Arg include nitro, Tosyl (Tos), Cbz, adamantyloxycarbonyl mesitoylsulfonyl (Mts), or Boc. The side chain protecting groups for Lys include Cbz, 2-chlorobenzyloxycarbonyl (2-Cl-Cbz), 2-bromobenzyloxycarbonyl (2-BrCbz), Tos, or Boc.

After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. An excess of each protected amino acid is generally used with an appropriate carboxyl group activator such as dicyclohexylcarbodiimide (DCC) in solution, for example, in methylene chloride (CH_2Cl_2), dimethyl formamide (DMF) mixtures.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treatment with a reagent such as trifluoroacetic acid or hydrogen fluoride (HF), which not only cleaves the peptide from the resin, but also cleaves all remaining side chain protecting groups. When the chloromethylated resin is used, hydrogen fluoride treatment results in the formation of the free peptide acids. When the benzhydrylamine resin is used, hydrogen fluoride treatment results directly in the free peptide amide. Alternatively, when the chloromethylated resin is employed, the side chain protected peptide can be decoupled by treatment of the peptide resin with ammonia to give the desired side chain protected amide or with an alkylamine to give a side chain protected alkylamide or dialkylamide. Side chain protection is then removed in the usual fashion by treatment with hydrogen fluoride to give the free amides, alkylamides, or dialkylamides.

In preparing the compounds of the invention, the resins used to prepare the peptide acids are employed, and the side chain protected peptide is cleaved with base and the appropriate alcohol, i.e., methanol. Side chain protecting groups are then removed in the usual fashion by treatment with hydrogen fluoride to obtain the desired ester. These solid phase peptide synthesis procedures are well known in the art and further described in Stewart, SOLID PHASE PEPTIDE SYNTHESES (Freeman and Co., San Francisco, CA, 1969).

The peptides and peptidomimetics typically are synthesized as the free acid but, as noted above, can be readily prepared as the amide or ester. One can also modify the amino and/or carboxy terminus of the peptide compounds of the invention to produce other compounds of the invention. Amino terminus modifications include methylating (i.e., $-N(CH_3)_2$ or $-NH(CH_3)$), acetylating, adding a carbobenzyloxy group, or blocking the amino terminus with any blocking group containing a carboxylate functionality defined by RCOO-, where R is selected from the group consisting of naphthyl, acridinyl, steroidyl, and similar groups. Carboxy terminus modifications include replacing the free acid with a carboxamide group or forming a cyclic lactam at the carboxy terminus to introduce structural constraints. One can also cyclize the peptides of the invention, or incorporate a desamino or descarboxy residue at the termini of the peptide, so that there is no terminal amino or carboxyl group, to decrease susceptibility to proteases or to restrict the conformation of the peptide. C-terminal functional groups of the compounds of the present invention include amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, and carboxy, and the lower ester derivatives thereof, and the pharmaceutically acceptable salts thereof.

E. *In Vitro* Uses

The compounds of the invention are useful *in vitro* as unique tools for understanding the biological role of SH2 domains, including the evaluation of the many factors thought to influence, and be influenced by, the cellular signal transduction pathway. The present compounds are also useful in the development of other compounds that bind to SH2 domains because the present compounds provide important information on the relationship between structure and activity that should facilitate such development.

The compounds are also useful as competitive inhibitors in assays to screen for new compounds that bind to SH2 domains. In such assay embodiments, the compounds

of the invention can be used without modification or can be modified in a variety of ways; for example, by labeling, such as covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, the materials thereto can be labeled either directly or indirectly. Possibilities for direct labeling include label groups such as: radiolabels such as ^{125}I, enzymes (U.S. Patent No. 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (U.S. Patent No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups. The compounds may also include spacers or linkers in cases where the compounds are to be attached to a solid support.

Thus, the compositions and methods of the present invention also can be used *in vitro* for testing a patient's susceptibility to varying treatment regimens for disorders having symptoms related to aberrant cell growth, differentiation, or regulation associated with defects in receptor tyrosine kinase pathways using an *in vitro* diagnostic method whereby a specimen is taken from the patient and is treated with a SH2 binding peptide compound of the present invention to determine the effectiveness and amount of the compound necessary to produce the desired effect. The SH2 binding peptide compounds are screened, then the appropriate treatment and dosage can be selected by the physician and administered to the patient based upon the results. Therefore, this invention also contemplates use of a SH2 binding peptide compound of this invention in a variety of diagnostic kits and assay methods.

F. *In Vivo* Uses

The compounds of the invention can also be administered to warm blooded animals, including humans, to block at least partially the signal transduction pathways in cells expressing SH2 domains *in vivo*. Thus, the present invention encompasses methods for therapeutic treatment of SH2 related disorders that comprise administering a compound of the invention in amounts sufficient to at least partially block or inhibit the signal transduction pathway involving SH2 domains *in vivo*. For example, the peptides and compounds of the invention can be administered to treat symptoms related to aberrant cell growth, differentiation or regulation which is associated with defects in receptor tyrosine kinase pathways.

Accordingly, the present invention includes pharmaceutical compositions comprising, as an active ingredient, at least one of the peptides or other compounds of the invention in association with a pharmaceutical carrier or diluent. The compounds of this invention can be administered by oral, parenteral (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous injection), nasal, vaginal, rectal, or sublingual routes of administration and can be formulated in dosage forms appropriate for each route of administration.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as is normal practice, additional substances other than inert diluents, *e.g.*, lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, with the elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

Preparations according to this invention for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized by, for example, filtration through a bacteria retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured using sterile water, or some other sterile injectable medium, immediately before use.

Compositions for rectal or vaginal administration are preferably suppositories which may contain, in addition to the active substance, excipients such as cocoa butter or

a suppository wax. Compositions for nasal or sublingual administration are also prepared with standard excipients well known in the art.

"Pharmaceutically acceptable carrier" refers to either solid or liquid material, which may be inorganic or organic and of synthetic or natural origin, with which the active component of the composition is mixed or formulated to facilitate administration to the subject. Any materials customarily employed in formulating pharmaceuticals are suitable. Solid carriers include natural and synthetic cloisonne silicates, for example, natural silicates such as diatomaceous earths; magnesium silicates for example, talcs; magnesium aluminum silicates, for example, attapulgites and vermiculites; aluminum silicates, for example, kaolinites, montmorillonites, and micas; calcium carbonate; calcium sulfate; synthetic hydrated silicone oxides and synthetic calcium or aluminum silicates; elements such as carbon or sulfur; natural and synthetic resins such as polyvinyl alcohol; and waxes such as paraffin and beeswax. Examples of suitable liquid carriers include water and aqueous solutions containing oxygenated organic compounds such as ethanol. Buffers and other materials normally present in pharmaceutical preparations, such as flavoring and suspending agents, can also be present. Pharmaceutical carriers differ from typical solutions and suspensions in that they are specifically prepared for use *in vivo* to exclude substances that may be harmful to the host to whom the composition is administered (*e.g.*, removal of bacterial toxins).

In general, water, a suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions. Solutions for parenteral administration preferably contain a water soluble salt of the active ingredient, suitable stabilizing agents, and if necessary, buffer substances. Antioxidizing agents such as sodium bisulfite, sodium sulfite, or ascorbic acid, either alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts and sodium EDTA. In addition, parenteral solutions can contain preservatives, such as benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol.

Suitable pharmaceutical carriers are described in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Co., a standard reference text in this field.

The quantities of the SH2 binding peptide necessary for effective therapy will depend upon many different factors, including means of administration, target site,

physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, *e.g.*, in Gilman *et al.* (eds.) (1990) GOODMAN AND GILMAN'S: THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 8th ed., Pergamon Press; and REMINGTON'S PHARMACEUTICAL SCIENCES (1985), 7th ed., Mack Publishing Co., Easton, PA, each of which is hereby incorporated by reference.

The dosage of active ingredients in the compositions of this invention may be varied; however, it is necessary that the amount of the active ingredient shall be such that a suitable dosage form is obtained. The selected dosage depends upon the desired therapeutic effect, on the route of administration, and on the duration of the treatment desired. It is expected that dosage levels of between 0.001 to 10 mg/kg of body weight daily can be administered to mammals to obtain effective SH2 blocking activity.

It should, of course, be understood that the compositions and methods of this invention can be used in combination with other agents exhibiting the ability to modulate signal transduction pathways or block the binding to SH2 domains. Examples of such agents include, but are not limited to those described in WO 94/07913, published April 14, 1994 by Dobrusin *et al.*; Domchek *et al.*, *Biochem.*, **31**:9865-9870 (1992); Burke *et al.*, *J. Med. Chem.*, **34**:1577-1581 (1991); Burke *et al.*, *Biochem.*, **33**:6490-6494 (1994).

In addition, these agents can be covalently attached, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the SH2 binding peptides of the present invention. Such non-interfering positions generally are positions that do not form direct contacts with those portions of the SH2 domains to which the SH2 binding peptides binds to produce the therapeutic effect. Since the covalent attachment of these therapeutic agents should not substantially interfere with the ability of the SH2 binding peptides to bind to SH2 domains, the SH2 binding peptides serve as a means to direct the therapeutic agents to the SH2 domains. Also, the invention embraces the use of SH2 polymers, wherein the repeating unit comprises a peptide of the present invention or a fragment thereof.

The compositions containing the compounds can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient.

In prophylactic applications, compositions containing the compounds of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight.

EXAMPLES

The examples below, provide the preparation of monomeric amino acids and their isosteres. Other examples describe the use of these monomers to prepare libraries of peptides on solid supports (both bead-based and on chips) as well as individual peptides and derivatives. The screening of the libraries and testing of individual peptides are carried out using the assays described.

A. Monomers

The monomers used in the preparation of the libraries and peptides described below are either commercially available or can be prepared by the methods herein. The monomers used are typically in protected form and are gene-encoded amino acids, non-gene-encoded amino acids, phosphotyrosine isosteres and asparagine isosteres. Additionally, for the purposes of peptide design, the gene-encoded and non-gene-encoded amino acids have been grouped as aromatic amino acids (19), basic amino acids (8), acidic amino acids (7), backbone modifying amino acids (17), neutral hydrogen-bonding amino acids (23), hydrophobic and aliphatic amino acids (17), lysine derivatives (19), D-amino acids (19) and L-amino acids (19). **Figure 12** provides the structures of a family of 36 amino acids which were used for ESL constructions. **Figures 13(a-i)** provide the structures for members of the individual classes noted above.

1. Gene-Encoded and Non-Gene-Encoded Amino Acids

Example 1
Synthesis of Building Blocks for ESL
Protection of Amino Acids

Amino acids **1-9** (in **Figure 14**) were prepared from unprotected amino acids for ESL studies. Monomers **1-5** were protected using FmocOPfp/DIEA protocol, whereas amino acids **6-9** were preferably prepared with BSTFA/Fmoc-Cl/DIEA (isolated yields were in the range of 60-80%). Compound **8** was synthesized by subsequent tritylation of the corresponding N-Fmoc amino acid with Trt-OH in $AcOH/Ac_2O$ in the presence of catalytic amounts of sulfuric acid (50°C, 6 hours; isolated yield 79%). Compounds **8** and **9** were prepared in amounts of ~3.0 and 3.5 g, respectively.

2. Phosphotyrosine Isosteres

A number of phosphotyrosine isosteres have been described in the literature including those described in WO 94/11392, by Dobrusin, published on May 26, 1994; WO 94/07913 by Dobrusin et al., published on April 14, 1994; Burke, *et al.*, *Biochemistry*, **33**:6490-6494 (1994); Burke, *et al.*, *J. Med. Chem.*, **34**:1577-1581 (1991) which specific compounds are methods of synthesis are incorporated by reference herein. Specific examples of phosphotyrosine analogs and isosteres include by way of example tyr($CH_2PO_3H_2$), tyr(SO_3H), tyr(CH_2CO_2tBu), tyr(CH_2CO_2OH), tyr($CH_2PO_3Et_2$), 4-phosphono(difluoromethyl)-L-phenylalanine (F_2Pmp), N-Fmoc α-Me O-phosphotyrosine, AcF_2Pmp, Fmoc-D-tyr(PO_3H_2)OH, Fmoc-(α-Me) tyr(PO_3H_2)-OH, Fmoc-L-Tyr(CH_2CO_2tBu)-OH, Ac-L-Phe($CH_2PO_3Bu_2$)-OH, N^{α}-Fmoc-L-Tyr($CH_2PO_3H_2$)-OH, N^{α}-Cbz-Tyr($CH_2PO_3Et_2$)-OH, N^{α}-Cbz-L-Tyr($CH_2PO_3Et_2$)-OMe, L-Tyr($CH_2PO_3Et_2$)-OH, N^{α}-Fmoc-L-Tyr($CH_2PO_3Et_2$)-OH, N^{α}-Fmoc-L-Tyr($CH_2PO_3H_2$)-OH and MeNpoc-L-Tyr(PO_3H_2)-OH.

These and other tyrosine and phosphotyrosine analogs can be substituted for tyrosine or phosphotyrosine in the SH2 binding peptides of the invention or those known in the art. Representative syntheses of the phosphotyrosine isosteres noted above are provided. Examples 2-3 illustrate the synthesis of phosphonic acid and carboxylic acid analogs of phosphotyrosine (pY). Example 4 illustrates the synthesis of a fluorinated analog of pY. Examples 5-9 illustrate the preparation of compounds which are malonylmethylene analogs of pY. Further modifications to desamino derivatives are provided in Examples 10-13. The synthesis of conformationally restricted analogs of pY are provided in Examples 14-17. The preparation of nucleic acid derivatives as pY isosteres is provided in Examples 18-19.

EXAMPLE 2

This example illustrates the preparation of N^{α}-Fmoc-L-Tyr($CH_2PO_3H_2$)-OH, shown schematically in **Figure 15**.

Preparation of N^{α}-Cbz-L-Tyr($CH_2PO_3Et_2$)-OMe, 10.

N^{α}-Cbz-L-Tyr-OMe (5.0 g, 15.18 mmol) was added at 0°C to a suspension of sodium hydride (95%, 0.422 g, 16.70 mmol) in dry THF (40 mL), and stirred for 10 min. Diethyl phosphonomethyltriflate (5.92 g, 19.73 mmol, *see* Phillion, *et al.*, *Tetrahedron Lett.*, **27**:1477 (1986)) was added rapidly. The reaction was stirred for 30 min at 0°C and 30 min at room temperature. The reaction mixture suspended in ethyl acetate (600 mL), washed with water (40 x 2 mL) and saturated aqueous sodium chloride (40 x 2 mL), dried over $MgSO_4$, filtered, concentrated, then purified by chromatography on silica (eluting with 2:3 ethyl acetate:hexane and 1:1 MeOH:ethyl acetate) to give **10** (5.51 g, 76%) as an oil. TLC: R_f = 0.29 (5:1 ethyl acetate:hexane, visualized by UV and PMA); MS: $(M+1)^+$ 480.

Preparation of N^{α}-Cbz-L-Tyr($CH_2PO_3Et_2$)-OH, 11.

A 1 N solution of aqueous sodium hydroxide (1.9 mL, 1.9 mmol) was added dropwise to the solution of N^{α}-Cbz-L-Tyr($CH_2PO_3Et_2$)-OMe in p-dioxane (10 mL) at 0°C. After stirring for 10 min at 0°C and 1 h at room temperature, the mixture was concentrated to half of its volume, and was acidified with 1 N HCl to pH ~2-3, extracted with ethyl acetate (3 x 150 mL), dried over $MgSO_4$ and concentrated to give N^{α}-Cbz-L-Tyr($CH_2PO_3Et_2$)-OH (0.775 g, 88%) as a solid. TLC: R_f = 0.50 (65:5:5 $CHCl_3$:MeOH:HOAc, visualized UV and PMA); MS: $(M+1)^+$ 466

Preparation of L-Tyr($CH_2PO_3Et_2$)-OH, 12.

A solution of N^{α}-Cbz-L-Tyr($CH_2PO_3Et_2$)-OH (0.746 g, 1.604 mmol) in EtOH (10 mL) and 20% $Pd(OH)_2$ (75mg) was hydrogenated (H_2 balloon) for 3.5 h at room temperature. The catalyst was filtered, washed with EtOH, the filtrates were combined, and concentrated under vacuum to afford L-Tyr($CH_2PO_3Et_2$)-OH (0.439 g, 83%) as a solid. MS: $(M+1)^+$ 332.

Preparation of N^{α}-Fmoc-L-Tyr($CH_2PO_3Et_2$)-OH, 13

Trimethylsilylchloride (0.34 mL, 2.66 mmol) was added in one portion to a suspension of L-Tyr($CH_2PO_3Et_2$)-OH (0.439 g, 1.326 mmol) in CH_2Cl_2 (6 mL) and dioxane (7 mL). After cooling to 0°C, DIEA (0.59 mL, 2.52 mmol) and Fmoc-Cl

(0.326 g, 1.26 mmol) were added. The solution was stirred for 15 min at 0°C and warmed to room temperature for 1 h. The mixture was concentrated, and purified by chromatography on silica gel (200:10:1 ethyl acetate:MeOH:HOAc as eluant) to provide N^{α}-Fmoc-L-Tyr($CH_2PO_3Et_2$)-OH (0.635 g, 87%) as a solid.
TLC: R_f = 0.53 (65:5:5 $CHCl_3$:MeOH:HOAc, visualized by UV and PMA);
MS:$(M+1)^+$ 554

Preparation of N^{α}-Fmoc-L-Tyr($CH_2PO_3H_2$)-OH, 14.

A solution of N^{α}-Fmoc-L-Tyr($CH_2PO_3Et_2$)-OH (0.617 g, 1.11 mmol) in anhydrous CH_2Cl_2 (30 mL) and TMS-Br (10 mL) was stirred overnight at room temperature. After removal of solvent and excess TMS-Br, the mixture was stirred with MeOH for 30 min, to afford the desired compound, N^{α}-Fmoc-L-Tyr($CH_2PO_3H_2$)-OH (0.479 g, 86%) as white solid. MS: $(M+1)^+$ 498.

EXAMPLE 3

This example illustrates the preparation of Fmoc-L-Tyr(CH_2CO_2tBu)-OH, shown schematically in **Figure 16**.

Preparation of Cbz-L-Tyr(CH_2CO_2tBu)-OH, 15.

A mixture of Cbz-L-Tyr-OH (0.3 g, 0.95 mmol) and NaH (95%, 53 mg, 2.09 mmol) in anhydrous DMF (6 mL) was stirred for 1 h at room temperature. Tert-butyl bromoacetate (0.16 mL, 0.99 mmol) was added, and the resulting solution was stirred an additional 12 h at room temperature. After removal of the DMF under vacuum, the residue was diluted with 5% aqueous sodium bicarbonate (10 mL), washed with hexane (15 x 2 mL), acidified with 2 N HCl, extracted with ethyl acetate (25 x 3 mL), washed with 10% aqueous LiCl (20 x 2 mL), dried over $MgSO_4$, filtered, and then concentrated under the vacuum, to provide Cbz-L-Tyr(CH_2CO_2tBu)-OH (0.365 g, 89%) as sticky solid. TLC: R_f = 0.42 (15:1 EtOAc:MeOH with HOAc, visualized UV and PMA); MS: $(M+1)^+$ 430.

Preparation of L-Tyr(CH_2CO_2tBu)-OH, 16.

A solution of Cbz-L-Tyr(CH_2CO_2tBu)-OH (1.0 g, 2.33 mmol) in methanol (15 mL) and 20% $Pd(OH)_2/C$ (0.2g) was hydrogenated (balloon) for 5 h. The mixture was filtered and concentrated under vacuum to afford L-Tyr(CH_2CO_2tBu)-OH (0.64 g, 93%) as an oil. TLC: R_f = 0.5 (5:1:1 BuOH:H_2O:HOAc visualized UV and ninhydrin); MS: $(M+1)^+$ 297.

Preparation of Fmoc-L-Tyr(CH_2CO_2tBu)-OH, 17.

Trimethylsilylchloride (0.55 mL, 4.32 mmol) was added in one portion to a suspension of L-Tyr(CH_2CO_2tBu)-OH (0.64 g, 2.16 mmol) in CH_2Cl_2 (10 mL) and dioxane (5 mL). Diisopropylethylamine (0.96 mL, 4.10 mmol) and Fmoc-Cl (0.559 g, 2.16 mmol) were then added at 0°C. The solution was stirred for 20 min at 0°C and warmed to room temperature for 2 h. The mixture was concentrated, and the residue was partitioned between 5% aqueous sodium bicarbonate (20 mL) and hexane-ether (15 x 2 mL). The aqueous layer was acidified to pH = 2 with 2 N HCl and extracted with ethyl acetate (40 mL x 3), dried over $MgSO_4$, filtered and concentrated under vacuum. Silica gel chromatography provided Fmoc-L-Tyr(CH_2CO_2tBu)-OH (0.602 g, 54% yield) as a solid. TLC: R_f = 0.55 (65:5:5 $CHCl_3$:MeOH:HOAc, visualized by UV and PMA); MS: $(M+1)^+$ 517.

EXAMPLE 4

This example illustrates the preparation of 4-((2-t-Butoxycarbonyl-2,2-difluoro)-ethyl)-N-Fmoc-phenylalanine, shown schematically in **Figure 17**.

Preparation of compound 18.

Ethyl bromodifluoroacetate (5.7 g, 28 mmol) was added to a refluxing suspension of activated zinc dust (1.84 g, 28 mmol) in tetrahydrofuran (50 mL). After the vigorous reaction had subsided (2 min), the chloromethylbenzaldehyde (3.63 g, 24 mmol) was added in a small volume of tetrahydrofuran (~4mL). After 15 min of reflux the reaction mixture was allowed to cool to room temperature, quenched with ethyl acetate (70 mL) and washed with saturated sodium chloride and 5% sodium thiosulfate solutions. The ethyl acetate solution was dried over sodium sulfate, filtered and the solvent removed by rotary evaporation. The resulting residue was triturated with hexane to give the desired hydroxy ester **18** (5.6 g, 84%). ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure. TLC: R_f = 0.29 (methylene chloride)

Preparation of compound 19.

Thiocarbonyldiimidazole (TCDI, 1.2 g, 6.6 mmol) was added to a solution of the hydroxy ester **18** (1.81 g, 6.6 mmol) in 1,2-dichloroethane (18 mL) . After stirring at 70°C for 1 h the reaction mixture was diluted with methylene chloride (50 mL), washed with water, dried (sodium sulfate) and the solvent removed via rotary evaporation. After plug filtration through silica gel with 5% ethyl acetate/methylene chloride, the resulting thiocarbamate was dissolved in benzene (40 mL) followed by the addition of tri-n-butyltin hydride (2.1 mL, 7.8 mmol). The resulting mixture was refluxed for 1 h, cooled, diluted with ether, washed with 1 N hydrochloric acid, dried (sodium sulfate) and the solvent removed via rotary evaporation. The resulting oil was purified by column chromatography (silica gel, 50/50 hexane/methylene chloride) to give the desired ester **2** (2.05 g, 78%). ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure. TLC: R_f = 0.36 (50:50 hexane:methylene chloride)

Preparation of compound 20.

A mixture of the ester **19** (1.05 g, 0.004 mol), concentrated hydrochloric acid (5 mL) and glacial acetic acid (5 mL) was rapidly stirred at 100°C for 30 min, cooled and then rotary evaporated to give a white residue. The residue was suspended in toluene (50 mL) and the mixture rotary evaporated to dryness. After repeating this once more the solid residue was vacuum dried. The crude acid was then dissolved in *t*-butyl acetate (25 mL) and 70% perchloric acid (1 drop) was added. After stirring overnight at room temperature the reaction mixture was diluted with ethyl acetate (60 mL) and then washed with saturated sodium bicarbonate. The organic layer was then dried (sodium sulfate) and the solvent removed to give the desired ester **20** (620 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure. TLC: R_f = 0.94 (CH_2Cl_2, visualized by PMA)

Preparation of compound 21.

A mixture of the ester **20** (520 mg, 1.8 mmol) and sodium iodide (406 mg, 2.71 mmol) in acetone (20mL) was heated to 60-70°C for 3 h. The mixture was then cooled and diluted with ethyl acetate (60 mL). The mixture was washed with water, 5% sodium thiosulfate, dried (sodium sulfate) and the solvent removed via rotary evaporation to give the desired iodo ester **21** (570 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure. TLC: R_f = 0.94 (methylene chloride, visualized by PMA).

Preparation of compound 22.

A solution of lithium bis(trimethylsilyl)amide in tetrahydrofuran (1.6 mL, 1.6 mmol) was added dropwise to a solution of the iodide **21** (600 mg, 1.6 mmol), the lactone (561 mg, 1.45 mmol) and hexamethyl phosphoramide (3.5 mL) in tetrahydrofuran (35 mL) at -78°C. After stirring for 45 min at -78°C, ethyl acetate (30 mL) was added and the mixture was washed with water, saturated sodium chloride, dried (sodium sulfate) and the solvent removed via rotary evaporation. Column chromatography of the crude product afforded the desired alkylated lactone **22** (322 mg, 35%).

^{1}H NMR (400 MHz,$CDCl_3$): Consistent with proposed structure. TLC: R_f = 0.77 (5% ethyl acetate/methylene chloride, visualized by PMA)

Preparation of compound 23.

The alkylated lactone **22** (344 mg, 0.54 mmol) in a small volume of methanol was added to a suspension of palladium (freshly prepared by reduction of palladium dichloride (60 mg) with hydrogen) in ethanol (2 mL) and tetrahydrofuran (1 mL). After vacuum aspiration to remove air the flask was filled with hydrogen at 50 psi and the mixture was stirred for 20 h. The reaction was then filtered through celite and the filtrate rotary evaporated to dryness. The residue was then triturated three times with ether and dried under vacuum to give the desired amino acid **23** (130 mg, 78%).
^{1}H NMR (400 MHz,$CDCl_3$): Consistent with proposed structure.

Preparation of compound 24.

Dioxane (2 mL) was added to a solution of the amino acid **23** (120 mg, 0.365 mol) and sodium bicarbonate (41 mg) in water (2 mL). The mixture was then cooled in an ice bath and then treated with Fmoc-N-hydroxysuccinimide (164 mg, 0.481 mmol) in a small amount of dioxane. After stirring for 3 h at room temperature, the reaction mixture was diluted with saturated sodium bicarbonate (10 mL) and then extracted with ether. The aqueous layer was acidified to pH 2 with 6 N hydrochloric acid and extracted with ethyl acetate. The extracts were dried (sodium sulfate) and the solvents removed yielding a white solid. Column chromatography with 10% methanol/methylene chloride afforded the desired Fmoc protected amino acid **24** (150 mg, 80%). mp 240-250°C decomposed; MS: $(M+Na)^+$ 574.3; ^{1}H NMR (400 MHz,$CDCl_3$): Consistent with proposed structure.

EXAMPLE 5

This example illustrates the preparation of 4-(2-Cyano-2-(t-butoxycarbonyl)-ethenyl)-N-acetyl-phenylalanine, shown schematically in **Figure 18**.

Preparation of Ac-L-Phe(p-CH_2Cl)-OEt, 25.

The title compound was prepared according to the methods reported in *J. Med. Chem.*,*36*, 1681-1688 (1993).

Preparation of 26.

A solution of compound **25** (994 mg, 3.5 mmol) and methenamine (981.4 mg, 7 mmol) in a mixture of acetic acid (3 mL) and water (3 mL) was heated in an oil bath at 110°C for 2 h and then the temperature of the oil bath was lowered to 90°C. Hydrochloric acid (12 N, 1 mL) was added and the mixture was heated for 30 min then cooled to room temperature. The solution was diluted with deionized water (15 mL) and extracted with dichloromethane (2 x 50 mL). The organic layers were combined and washed with saturated aqueous sodium chloride (3 x 100 mL) until the pH of the washings became neutral. The dichloromethane solution was dried and concentrated *in vacuo* and triturated with hexane to yield **26** as white solid (799 mg, 3.03 mmol, 86.7%). mp 65-72°C; TLC: R_f = 0.22 (60:40:4 hexane:ethyl acetate:methanol, developed twice); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 27.

A catalytic amount of piperidine (3 mg) was added to a mixture of **26** (301 mg, 1.14 mmol) and t-butyl cyanoacetate (150 mg, 1.06 mmol) in benzene (1 mL) and the mixture was stirred at 35°C for 48 h. The mixture was then concentrated and purified by flash silica gel column chromatography, eluting with 40:60:1.5 ethyl acetate:hexane:methanol, to yield **27** as an oil (364 mg, 0.94 mmol, 88%). TLC: R_f = 0.47 (10:10:1 ethyl acetate:hexane:methanol); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 28.

Lithium hydroxide (1 N, 1.14 mL) was added to compound **27** (364 mg, 0.94 mmol) in p-dioxane (12 mL). The reaction mixture was stirred at room temperature for 3 h and then concentrated to dryness. The residue was redissolved in deionized water (10 mL) and the pH was adjusted to 3 by addition of 1 N hydrochloric acid. The mixture was extracted with dichloromethane (2 x 30 mL) and the organic layers were combined

and washed with saturated aqueous sodium chloride, dried and concentrated to yield crude product (255 mg) which was further purified by flash silica gel column. Elution with 100:10:0.4 dichloromethane:methanol:acetic acid yielded pure **28** (135 mg, 0.377 mmol, 40.1%). mp >260°C (decomposed); TLC: R_f = 0.33 (1:10 methanol:dichloromethane, developed twice); MS: $(M+Na)^+$ 381.1, $(M+H)^+$ 359.2; ^1H NMR (400 MHz, DMSO-d_6): Consistent with proposed structure.

EXAMPLE 6

This example illustrates the preparation of 4-(2,2-di-(t-butoxycarbonyl)-ethenyl)-N-acetyl-phenylalanine, shown schematically in **Figure 19**.

Preparation of 29.

A solution of compound **26** (810 mg, 3.076 mmol) and di-*tert*-butyl malonate (1.34 g, 6.2 mmol) in pyridine (3 mL) was added to piperidine (85 mg, 1 mmol) and molecular sieves 4Å (~ 1mL in volume) and heated at 100°C for 3 h TLC indicated some of compound **3** had not yet reacted. Additional piperidine (80 mg, 0.94 mmol) and molecular sieves 4Å (1 mL) were added and the mixture heated for an additional 3 h, until all **26** was converted. The molecular sieves were removed by filtration and rinsed several times with warm ethyl acetate. The filtrates were concentrated *in vacuo* to a crude oil (2.55 g) which was redissolved in ethyl acetate (70 mL) and washed with 0.5 N hydrochloric acid and saturated aqueous sodium chloride and dried. Final purification was achieved with flash silica gel column chromatography. Eluting with 40:60:2 ethyl acetate:hexane:methanol gave pure **29** (1.072 g, 2.32 mmol, 75.4%). TLC: R_f = 0.51 (40:60:4 ethyl acetate:hexane:methanol developed twice); ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 30.

Lithium hydroxide (0.5 N, 3 mL) was added to **29** (507 mg, 1.1 mmol) in p-dioxane (16 mL). The reaction mixture was stirred at room temperature for 1 h. TLC revealed all starting material had disappeared. The reaction mixture was concentrated *in vacuo* and the residue was redissolved in deionized water (25 mL). The pH was adjusted to 2 to 3 by addition of 0.5N hydrochloric acid and the solution was extracted with dichloromethane (3 x 50 mL). The organic layer was dried and concentrated. The residue was triturated with ether/hexane to yield **30** (334 mg, 0.77 mmol, 70.0%). mp 175-176.5°C; MS: $(M+H)^+$ 434.2; ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

EXAMPLE 7

This example illustrates the preparation of 4-(E-2-acetyl-2-(t-butoxycarbonyl)ethenyl)-N-acetyl-phenylalanine and 4-(2-acetyl,2-(t-butoxycarbonyl)-ethyl)-N-acetyl-phenylalanine, shown schematically in **Figure 20**.

Preparation of 31.

Molecular sieves 4Å (1 mL) and a catalytic amount of piperidine (5 mg) were added to a mixture of **26** (335 mg, 1.27 mmol) and t-butyl acetoacetate (200 mg, 1.27 mmol) in benzene (5 mL) and the mixture was stirred at room temperature for 72 h. The mixture was filtered and the filtrate was diluted with ether (20 mL) and washed with 20% aqueous sodium bisulfite solution, concentrated and purified by flash silica gel column chromatography, eluting with 40:60:2 ethyl acetate:hexane:methanol to yield **31** as an oil (300 mg, 0.743 mmol, 58.5%). TLC: R_f = 0.40 (50:50:2 ethanol:hexane:methanol); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 32.

Lithium hydroxide (1N, 1 mL) was added to a solution of **31** (300 mg, 0.743 mmol) in dioxane (12 mL) at room temperature. After stirring at room temperature 1 h, TLC indicated total disappearance of the starting material. 10% aqueous citric acid (2 mL) was added and the reaction mixture was concentrated to dryness. The residue was redissolved in dichloromethane (50 mL) and washed with 10% aqueous citric acid, saturated aqueous sodium chloride, dried and concentrated to yield a fluffy solid (240 mg). Trituration of the solid with hexane gave pure **32** (212 mg, 0.565 mmol, 76%). mp 87°C (foam); MS: $(M+H)^+$ 376.1; ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 33.

tert-Butyl acetoacetate (608 g, 3.84 mmol) in dimethylformamide (1 mL) was addded to a flask containing a suspension of sodium hydride (92 mg, 3.84 mmol) in dimethylformamide (3 mL) under argon. The reaction mixture was stirred at room temperature for 30 min, cooled to 0°C and added to a solution of **25** (446 mg, 1.57 mmol) in dimethylformamide (1 mL) at 0°C. The reaction mixture was stirred at 0°C for 1 h and room temperature for 1.5 h. Saturated aqueous ammonium chloride (20 mL) was added and the mixture extracted with ethyl acetate (2 x 30 mL). Organic layers were

combined and washed with saturated aqueous sodium chloride (30 mL), dried and concentrated *in vacuo* to give crude oily material (720 mg). Flash column chromatography, eluting with 2:5 ethyl acetate:dichloromethane, gave a major fraction of crude **33** (441 mg) containing 20% of unreacted **25**. The crude product was redissolved in dichloromethane (5 mL) and treated with ethylene diamine (63 mg) at room temperature for 17 h. The reaction mixture was concentrated *in vacuo* and the residue poured into 10% aqueous citric acid (20 mL) and extracted with ethyl acetate (2 x 30 mL). The organic layers were combined, washed with saturated aqueous sodium chloride, dried and concentrated to yield pure **33** (320 mg, 0.79 mmol, 52.7%). TLC: $R_f = 0.66$ (60:40:4 hexane:ethyl acetate:methanol); ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 34.

Lithium hydroxide (0.4 N, 5 mL) was added to a solution of **33** (320 mg, 0.789 mmol) in dioxane (12 mL) at room temperature. After stirring at room temperature 1 h, TLC indicated total disappearance of the starting material. 10% aqueous citric acid (5 mL) was added and the reaction mixture was concentrated to dryness. The residue was taken up with deionized water (5 mL) and extracted with ethyl acetate (2 x 20 mL). The combined ethyl acetate extract was washed with saturated aqueous sodium chloride, dried and concentrated to yield a fluffy solid of **34** (280 mg, 0.742 mmol, 94%). mp 105-110°C (foam); MS: $(M+Na)^+$ 400.2 and $(M+H)^+$ 378.3; ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

EXAMPLE 8

This example illustrates the preparation of N-Acetyl-L-4-(dimyristyl-malonylmethyl)-phenylalanine, shown schematically in **Figure 21**.

Preparation of compound 35.

Aqueous lithium hydroxide (1 N, 3.5 mL, 0.0035 mol) was added to a solution of (4-chloromethyl)-N-acetylphenylalanine ethyl ester (1 g, 0.0035 mol) in dioxane (14 mL). After stirring for 1 h at room temperature the mixture was diluted with water (30 mL) and extracted with ether. The aqueous layer was then acidified to pH 2 with 1 N hydrochloric acid and extracted with ethyl acetate. The extracts were dried (sodium sulfate) and the solvents removed to give the crude acid. The acid was dissolved in dimethylformamide (8 mL) to which was added cesium carbonate (1.4 g, 0.0042 mol) followed by benzyl bromide (0.72 g, 0.0042 mol). After stirring for 3 h at room

temperature the mixture was diluted with ethyl acetate (40 mL) and washed with 1 N hydrochloric acid, saturated sodium bicarbonate (3X) and water, dried (sodium sulfate) and the solvent removed by rotary evaporation to give the desired ester **35** (1.06 g, 88%). ^{1}H NMR (400MHz,$CDCl_3$): Consistent with proposed structure.

Preparation of compound 36.

Malonyl dichloride (1 g, 0.0071 mol) was added to a solution of myristyl alcohol (3.06 g, 0.0143 mol) in methylene chloride (15 mL). After a vigorous exotherm the reaction mixture was stirred at room temperature for 48 h. The reaction mixture was diluted with methylene chloride (40 mL) and washed with saturated sodium bicarbonate, dried (sodium sulfate) and the solvent removed to give the diester **36** (3.82 g, 100%). ^{1}H NMR(400 MHz,$CDCl_3$): Consistent with proposed structure.

Preparation of compound 37.

The diester **36** (1.2 g, 2.4 mmol) in DMF (5 mL) was added dropwise to a suspension of dry sodium hydride (60 mg, 2.4 mmol) in dimethylformamide (20 mL) at 0°C. After stirring at room temperature for 45 min, a homogeneous yellowish solution was obtained to which was added, at ice bath temperature, the protected chloromethyl phenylalanine **35** (750 mg, 2.17 mmol). The ice bath was removed and the mixture was stirred at 35°C overnight, poured into saturated ammonium chloride solution and extracted twice with ethyl acetate. The ethyl acetate fractions were dried (magnesium sulfate) and the solvent removed via rotary evaporation to give, after column chromatography with 5% methanol/methylene chloride, the desired alkylate **37** (1.1 g, 60%). ^{1}H NMR (400MHz,$CDCl_3$): Consistent with proposed structure. TLC: R_f = 0.9 (5% MeOH/methylene chloride, visualized by PMA).

Preparation of compound 38.

Palladium-on-carbon catalyst (10%, 300 mg) was added to a solution of the malonyl methyl compound **37** (800 mg, 0.994 mmol) in methanol (30 mL). The mixture was aspirated and the reaction flask filled with hydrogen. The mixture was stirred under a positive hydrogen atmosphere at room temperature for 1 h, filtered and the filtrate rotary evaporated to give the desired acid **38** (690 mg, 97%). TLC: R_f = 0.06 (5% MeOH/methylene chloride, visualized by PMA); MS: (M+Na) = 738.5; ^{1}H NMR (400MHz,$CDCl_3$): Consistent with proposed structure.

EXAMPLE 9

This example illustrates the preparation of 4-(N,N-Di-(t-butoxycarbonyl methyl)-amino)-N-acetyl-phenylalanine, shown schematically in **Figure 22**.

Preparation of 39.

A mixture of a N-acetyl-p-amino-phenylalanine methyl ester (512 mg, 2.16 mmol), *t*-butyl bromoacetate (1.208 g, 6.6 mmol) and 1,8-bis(dimethylamino)naphthalene (1.414 g, 6.6 mmol) in acetonitrile (8.6 mL) was heated in an oil bath at 75°C for 56 h. After cooling to room temperature, the precipitates were removed by filtration. The filtrate was poured into 10% aqueous citric acid solution (50 mL) and extracted with ethyl acetate (2 x 50 mL). The organic layer was washed with saturated aqueous sodium chloride (50 mL), dried and concentrated *in vacuo* to provide crude **39** (1.53 g). Flash silica gel column chromatography, eluting with 50:50:1 ethyl acetate:hexane:methanol, yielded pure **39** (948 mg, 2.04 mmol, 94.5%). TLC: R_f = 0.50 (50:50:1 ethyl acetate:hexane:methanol); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 40.

Lithium hydroxide (0.44 N, 5 mL) was added to a solution of **39** (848 mg, 2.04 mmol) in dioxane (20 mL) at room temperature. After stirring at room temperature 1 h, TLC indicated total disappearance of the starting material. The reaction mixture was concentrated to dryness and the residue was redissolved in deionized water (10 mL). 1 N hydrochloric acid was added until the pH became about 2 and the mixture was then extracted with dichloromethane (2 x 60 mL). The organic layer was washed with 10% aqueous citric acid (30 mL) and saturated aqueous sodium chloride (50 mL), dried and concentrated to yield **40** as a fluffy solid (886 mg, 1.967 mmol, 96.4%). mp 66-72°C; MS: $(M+Na)^+$ 472.9; ^{1}HNMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

EXAMPLE 10

This example illustrates the preparation of (E)-3-(4-(2,2-bis(t-butyloxy-carbonyl)ethyl)phenyl)propenoic acid and 3-(4-(2,2-bis(t-butyloxycarbonyl)ethyl)-phenyl)propanoic acid, shown schematically in **Figure 23**.

Preparation of 41.

Oxalyl chloride (1.62 mL, 18.5 mmol) was added in one portion to a suspension of 4-methyl-cinnamic acid (2.0 g, 13.8 mmol) in dichloromethane (15 mL) containing one drop of dimethylformamide. Then reaction mixture was stirred with gradual warming to room temperature After overnight at room temperature, the homogeneous reaction mixture was concentrated by rotary evaporation to remove dichloromethane. The acyl chloride thus obtained was stirred with methanol (15 mL) for 0.5 h at room temperature. TLC revealed mainly formation of ester. The excess methanol was removed *in vacuo* to give **41** as a yellowish white solid (2.05 g, 11.5 mmol). TLC: $R_f = 0.47$; ^1H NMR: (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 42.

Benzoyl peroxide (1.36 g, 5.64 mmol, 0.5 equivalents) and N-bromosuccinimide (1.8 g, 10.15 mmol, 0.9 equivalents) were sequentially added to a homogeneous mixture of methyl 4-methyl-cinnamate, **41** (2.0 g, 11.28 mmol) in carbon tetrachloride (40 mL). After refluxing for 1.5 h, TLC revealed mainly product (lower R_f than starting material). The reaction mixture was cooled to room temperature, filtered to remove the white solids and the filtrate was concentrated under vacuum to yield a yellow oil. The oil was redissolved in carbon tetrachloride and washed with 5% sodium bisulfite. The aqueous layer was extracted with carbon tetrachloride and the combined organic layers were washed with water and brine. Drying and concentration provided the crude product as a yellow oil (4.0 g.) which was purified by silica gel chromatography, eluting with 35% dichloromethane/hexane to yield pure **42** as a white solid (1.8 g., 63%). TLC: $R_f = 0.48$ (70% dichloromethane/hexane); ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 43.

To a homogeneous mixture of di-tert-butyl malonate (1.1 mL, 7.15 mmol) in dimethylformamide (5 mL) was added sodium hydride (171 mg, 7.15 mmol) in one portion under argon. After stirring at room temperature for 30 min, a homogeneous solution of **42** in dimethylformamide (2 mL) was added dropwise at 0°C. The yellow solution was stirred with gradual warming to room temperature and TLC revealed total disappearance of starting material after 3 h. The reaction mixture was quenched with saturated ammonium chloride (20 mL), extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were washed with brine. Drying and concentration provided crude **43** as a yellow oil (1.75 g) which was purified by silica gel chromatography,

eluting with 35% dichloromethane/hexane and then 50% dichloromethane/hexane, to yield pure **43** as a colorless oil (650 mg, 58%). TLC : R_f = 0.28 (70% dichloromethane/hexane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 44.

A solution of lithium hydroxide (17 mg, 1.5 equivalents) in water (1.6 mL) was added to a solution of **43** (110 mg, 0.281 mmol) in p-dioxane (4 mL). After overnight stirring at room temperature, TLC revealed total disappearance of starting material. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in water (5 mL) and acidified to pH 2.0 using 0.5 N hydrochloric acid. The acidic solution was extracted twice with dichloromethane. The combined organic extracts were dried and concentrated *in vacuo* to provide **44** as a white solid (70 mg, 66%). mp 170-172°C; 1H NMR (400MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 45.

10% palladium-on-carbon (162 mg) was added to a homogeneous solution of **43** (324 mg, 0.83 mmol) in methanol (5 mL). The mixture was degassed and hydrogenated at room temperature. TLC revealed total disappearance of starting material after 1 h. The catalyst was filtered through a pad of celite and the filtrate was concentrated to give pure product **45** (263 mg, 81%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 46.

A solution of lithium hydroxide (12.4 mg, 1.5 equivalents) in water (2.3 mL) was added to a solution of **45** (80 mg, 0.203 mmol) in p-dioxane (4 mL). After overnight stirring at room temperature, TLC revealed total disappearance of starting material. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in water (5 mL) and acidified to pH 2.0 using 0.5 N hydrochloric acid. The acidic solution was extracted twice with dichloromethane. The combined organic extracts were dried and concentrated *in vacuo* to provide **46** as a white solid (45 mg, 58%). ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

EXAMPLE 11

This example illustrates the preparation of (E)-3-(4-(2,2-bis(t-butyloxy carbonyl)ethenyl)phenyl)propenoic acid, shown schematically in **Figure 24**.

Preparation of 47.

Compound **42** (prepared as described in Example 10) (200 mg, 0.78 mmol), and methenamine (218 mg, 1.56 mmol) in a mixture of acetic acid (1 mL) and water (1 mL) was heated to 110°C in an oil bath. TLC revealed total disappearance of starting material after 2.5 h. The oil bath temperature was lowered to 90°C and concentrated hydrochloric acid (250 μL) was added. The mixture was heated for another 30 min at 90°C and cooled to room temperature. The solution was diluted with deionized water (7 mL) and extracted twice with dichloromethane. The organic extracts were combined and washed with water until the washings were neutral by pH paper. Drying and concentration provided the crude product which was then triturated with hexane to yield **47** as white solid (120 mg, 80%). TLC: R_f = 0.22 (20% ethyl acetate/hexane); ^{1}H NMR: (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 48.

Molecular seives 4A and a catalytic amount of piperidine (22.4 mg) were added to a homogeneous mixture of **47** (150 mg, 0.79 mmol) and di-tert-butyl malonate (341 mg, 1.57 mmol) in pyridine (1 mL). The reaction mixture was heated to 100°C and TLC revealed total disappearance of starting material after 6 h. The reaction mixture was filtered and the molecular sieves were washed with 5 x 40 mL hot ethyl acetate. The combined ethyl acetate layers were concentrated. The residual yellow oil was suspended in 0.5 N hydrochloric acid and extracted twice with ethyl acetate. The combined extracts were washed sequentially with water and brine. Drying and concentration provided the crude product which was then purified by silica gel chromatography, eluting with 10% ethyl acetate/hexane, to yield pure **48** (223 mg, 73%).

Preparation of 49.

A solution of lithium hydroxide (15.8 mg, 1.1 equivalents) in water (1 mL) was added to a solution of **48** (133 mg, 0.34 mmol) in p-dioxane (3 mL). After 1 h stirring at room temperature, TLC revealed total disappearance of starting material. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in water (5 mL) and acidified to pH 2.0 using 0.5 N hydrochloric acid. The acidic solution and associated white precipitates were extracted twice with dichloromethane. The combined organic extracts were dried and concentrated *in vacuo* to provide **49** as a white solid (126 mg, 100%). mp = 166-167°C; TLC: R_f = 0.24 (ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

EXAMPLE 12

This example illustrates the preparation of (E)-3-(4-(2,2-bis(t-butyloxy-carbonyl)ethenyl)phenyl)propanoic acid, shown schematically in **Figure 25**.

Preparation of 50.

10% palladium-on-carbon (2.5 g) was added to a homogeneous solution of 4-methyl-cinnamic acid (5.0 g, 30.8 mmol) in methanol (100 mL) and p-dioxane (50 mL). The mixture was degassed and hydrogenated at room temperature. TLC revealed total disappearance of starting material after 1.5 h. The catalyst was filtered through a pad of celite and the pad washed with methanol. The filtrate was concentrated to give pure product **50** as white solid (4.8 g, 95%). ^{1}H NMR (400 MHz, $CDCL_3$): Consistent with proposed structure.

Preparation of 51.

Oxalyl chloride (3.8 mL, 43.5 mmol) was added dropwise to a suspension of compound **50** (4.8 g, 29 mmol) in dichloromethane (50 mL) containing a few drops of dimethylformamide at 0°C. Then reaction mixture was stirred with gradual warming to room temperature. After 2 h at room temperature, the homogeneous reaction mixture was concentrated by rotary evaporation to remove dichloromethane. The acyl chloride thus obtained was stirred with methanol (50 mL) for 1 h at room temperature TLC revealed mainly formation of ester. The excess methanol was removed *in vacuo* to give **51** as a yellowish white solid (5.0 g, 100%). ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 52.

Benzoyl peroxide (2.2 g, 9.1 mmol, 0.5 equivalents) and N-bromosuccinimide (2.93 g, 16.8 mmol, 0.9 equivalents) was sequentially added to a homogeneous mixture of compound **51** (3.0 g, 16.8 mmol) in carbon tetrachloride (100 mL). After refluxing for 3 h, TLC revealed mainly starting material with formation of product and by-products. The reaction mixture was cooled to room temperature, filtered to remove the white solids and concentrated under vacuum to yield a yellow oil. The oil was redissolved in carbon tetrachloride and washed with 5% sodium bisulfite. The aqueous layer was extracted with carbon tetrachloride and the combined organic layers were washed twice with brine. Drying and concentration provided the crude product as a pinkish oil (6.2 g) which was purified by silica gel chromatography, eluting with 35% dichloromethane/hexane, 70% dichloromethane/hexane and dichloromethane, to yield

pure **52** as a white solid (740 mg, 4%). TLC: R_f = 0.63 (70% dichloromethane/hexane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 53.

Compound **52** (162 mg, 0.63 mmol) and methenamine (186 mg, 1.26 mmol) in a mixture of acetic acid (1.5 mL) and water (1.5 mL) were heated to 110°C in an oil bath. TLC revealed total disappearance of starting material after 2.5 h. The oil bath temperature was lowered to 90°C. and concentrated hydrochloric acid (250 μL) was added. The mixture was heated for another 30 min at 90°C and cooled to room temperature. The solution was diluted with deionized water (7 mL) and extracted twice with dichloromethane. The organic extracts were combined and washed with water until the washings were neutral by pH paper. Drying and concentration provided the crude product **53** as an oil (75 mg, 62.5%). TLC: R_f = 0.29 (20% ethyl acetate/hexane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 54.

Molecular seives 4Å and a catalytic amount of piperidine (10 mg, 0.11 mmol) were added to a homogeneous mixture of **53** (75 mg, 0.39 mmol) and di-*tert*-butyl malonate (169 mg, 0.78 mmol) in pyridine (1 mL). The reaction mixture was heated to 100°C and TLC revealed total disappearance of starting material after 6 h. The reaction mixture was filtered and the molecular sieves were washed with 5 x 40 mL hot ethyl acetate. The combined ethyl acetate layers were concentrated. The residual yellow oil was suspended in 0.5 N hydrochloric acid and extracted twice with ethyl acetate. The combined extracts were washed sequentially with water and brine. Drying and concentration provided the crude product which was then purified by silica gel chromatography, eluting with 10% ethyl acetate/hexane, to yield pure **54** as white solid (82 mg, 56%). TLC: R_f = 0.46 (20% ethyl acetate/hexane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 55.

A solution of 1 N lithium hydroxide (500 mL, 2.4 equivalents) was added to a solution of **54** (82 mg, 0.21 mmol) in p-dioxane (1.5 mL). After 3 h stirring at room temperature, TLC revealed total disappearance of starting material. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in water (5 mL) and acidified to pH 2.0 using 0.5 N hydrochloric acid. The acidic solution and the associated white precipitates were extracted twice with dichloromethane. The combined organic extracts were dried and concentrated *in vacuo* to provide **55** as a white solid (62 mg,

78%). TLC: R_f = 0.18 (ethyl acetate); ^{1}H NMR (400 MHz, CDCl3): Consistent with proposed structure.

EXAMPLE 13

This example illustrates the preparation of 3-(4-(Z-2-cyano-2-(t-butoxycarbonyl)ethenyl)phenyl)propionic acid, shown schematically in **Figure 26**.

Preparation of 47 and 56.

Oxalyl chloride (9.4 mL, 74.14 mmol) was addded in one portion to a suspension of 4-formyl-cinnamic acid (10 g, 46.76 mmol) in dichloromethane (130 mL) containing one drop of dimethylformamide. The reaction mixture was stirred at room temperature for 2 h and the solution became clear. Solvent was removed *in vacuo* and the acyl chloride obtained was stirred with methanol (50 mL) for 1 h at room temperature. TLC revealed two products were obtained. Excess methanol was removed *in vacuo*, and the residue was triturated with hexane. A white solid was isolated by filtration to yield pure **47** (7.40 g, 38.9 mmol). mp 83-84°C; TLC: R_f = 0.50 (dichloromethane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

The filtrate from above was concentrated to give an oil of relatively pure **56** containing a trace of **47** (2.6 g, 11.0 mmol, 19.4%). TLC: R_f = 0.33 (dichloromethane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Compound **56** (2.5 g, 10.6 mmol) in acetone (70 mL) was stirred with p-toluene sulfonic acid monohydride (50 mg) at room temperature for 1 h. The solvent was removed *in vacuo*, and the residue was redissolved in dichloromethane (50 mL) and washed with saturated aqueous sodium chloride. The organic layer was dried and concentrated to yield **47** (2.0 g, 99%).

Preparation of 57.

A mixture of **47** (6.4 g, 33.6 mmol), ethylene glycol (2.671 g, 43 mmol) and p-toluenesulfonic acid (56 mg) in benzene (90 mL) was refluxed for 17 h with a Dean-Stark trap to collect the water produced. The reaction mixture was concentrated *in vacuo* and the residue was dissloved in dichloromethane (70 mL) and washed with saturated aqueous sodium bicarbonate. The organic layer was dried and concentrated *in vacuo* to give crude **57** (8.7 g). Recrystalization in ether/hexane gave pure **57** (6.896 g, 29.43 mmol, 87.5%). mp 79-80°C; TLC: R_f = 0.31 (dichloromethane); ^{1}HNMR (400 MHz,$CDCl_3$): Consistent with proposed structure.

Preparation of 58.

Palladium-on-carbon (5%, 180 mg) was added to a solution of compound **57** (1.00 g, 4.27 mmol) in methanol (60 mL). The reaction mixture was cooled to 0°C, degassed, and stirred under a hydrogen atmosphere. The reduction was followed by checking the UV spectra of the reaction mixture. At 10 min intervals 5 mL samples were taken and diluted with methanol for UV measurement. After 1 h the absorption at 280 nm had all disappeared. Hydrogen was removed and the catalyst was removed by filtration. The filtrate was concentrated *in vacuo* to yield crude **58** (1 g) which was used for the preparation of **59** without further purification. TLC: R_f = 0.38 (dichloromethane); ^{1}H NMR: (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 59.

Crude **58** (3.20 g, 13.54 mmol) in acetone (70 mL) was stirred with p-toluene sulfonic acid monohydride (83 mg) at room temperature for 3 h. The solvent was removed *in vacuo*, and the residue was redissolved in dichloromethane (50 mL) and washed with saturated aqueous sodium chloride and saturated sodium bicarbonate. The organic layer was dried and concentrated to yield crude **59** (3.1 g). Flash silica gel chromatography, eluting with dichloromethane, yielded relatively pure **59** (2.4 g, 12.5 mmol, 92.2%). TLC: R_f = 0.48 (dichloromethane); ^{1}H NMR (400 MHz, $CDCL_3$): Consistent with proposed structure.

Preparation of 60.

Molecular sieves 4Å (1 mL) and a catalytic amount of piperidine (5 mg) were added to a mixture of **59** (384 mg, 2.0 mmol) and t-butyl cyanoacetate (290 mg, 2.05 mmol) in benzene (3.5 mL) and the mixture stirred at 35°C for 44 h. The mixture was concentrated and redissolved in dichloromethane (50 mL) and washed with 0.5 N hydrochloric acid (2 x 10 mL), dried and concentrated to give crude **60** (680 mg). Flash silica gel column chromatography, eluting with 40:60:1.5 ethyl acetate:hexane:methanol, yielded pure **60** (490 mg, 1.56 mmol, 77.7%) as a white solid. TLC: R_f = 0.55 (dichloromethane); ^{1}H NMR (400 M Hz, $CDCl_3$): Consistent with proposed structure.

Preparation of 61.

Lithium hydroxide (1 N, 1.7 mL) was added to compound **60** (490 mg, 1.56 mmol) in p-dioxane (5.1 mL). TLC revealed the formation of three polar compounds. The reaction mixture was stirred at room temperature for 1 h and 10% aqueous citric acid (4.5 mL) was added. The reaction mixture was concentrated *in vacuo* and extracted with dichloromethane (2 x 30 mL). The dichloromethane was

concentracted to give an oil (440 mg) which was redissolved in ethyl acetate (25 mL) and washed with 22% aqueous sodium bisulfite (20 mL, to remove the p-formyl-phenylpropionic acid produced) and saturated aqueous sodium chloride (20 mL), dried and concentrated to yield crude **61** (390 mg). Flash column chromatography, eluting with 3.5:100 methanol:dichloromethane, yielded pure **61** (97 mg, 0.322 mmol, 20.6%). mp 146-147°C; TLC: R_f = 0.26 (7:100 methanol:dichloromethane); MS: $(M+Na)^+$ 323.8; ^1H NMR (400 mHz, $CDCl_3$): Consistent with proposed structure.

EXAMPLE 14

This example illustrates the preparation of 2-(Di-(t-butoxycarbonyl)methyl)-6-naphthalenecarboxylic acid, shown schematically in **Figure 27**.

Preparation of 63.

Dimethyl 2,6-naphthalenedicarboxylate (**62**, 9.25 g, 37.87 mmol) in p-dioxane (500 mL) was stirred with aqueous lithium hydroxide (1.72 N, 22 mL) at room temperature for 110 h. The precipitate of the monoacid lithium salt was collected by centrifugation. The pellet was rinsed twice with tetrahydrofuran (2 x 60 mL) and centrifuged again. The pellet was triturated with 1 N hydrochloric acid (200 mL) and extracted with dichloromethane containing 6% methanol (5 x 500 mL). The organic layer was dried and concentrated *in vacuo* to provide **63** as a white solid (7.11 g, 30.89 mmol, 81.6%). mp 260-261°C; ^1H NMR (400HZ, $CDCl_3$ with one drop CD_3OD): Consistent with proposed structure; MS: $(M-H)^-$ 228.9.

Preparation of 64.

A solution of diborane in tetrahydrofuran (1 N, 12 mL) was added to a solution of monomethyl 2,6-naphthalenedicarboxylic ester (909 mg, 3.949 mmol) in tetrahydrofuran (20 mL) under argon at room temperature. After 1.5 h the solution became clear and stirring was continued for an additional 10 h. TLC revealed total disappearance of starting material. Water (5 mL) was added and the solution was concentrated *in vacuo*. The residue was partitioned between 1 N hydrochloric acid and dichloromethane (2 x 50 mL). The organic layers were combined and further washed with saturated aqueous sodium chloride (50 mL), saturated aqueous sodium bicarbonate (50 mL) and saturated aqueous sodium chloride (50 mL), dried and concentrated to provide crude **64** (910 mg). Compound **64** was further purified by silica gel chromaography, eluting first with dichloromethane then 3% methanol in dichloromethane,

to yield pure **64** (716 mg, 3.31 mmol, 83.3%). mp 120-121°C; TLC: R_f = 0.71 (dichloromethane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 65.

Dibromotriphenylphosphorane (1.862 g, 4.40 mmol) was added in one portion to a solution of **64** (736 mg, 3.41 mmol) in anhydrous acetonitrile (18 mL) at room temperature. After 1 h, TLC revealed all starting material was converted to a product with higher R_f. The reaction mixture was diluted with dichloromethane (100 mL) and washed sequentially with saturated aqueous sodium bicarbonate (50 mL), 5% aqueous sodium bisulfite (50 mL), saturated aqueous sodium chloride (50 mL), dried and concentrated to yield crude **65** (800 mg). Final purification of **65** with silica gel column chromatography, eluting with dichloromethane, gave pure **65** (716 mg, 2.57 mmol, 75.4%) as white needles. mp 104.5-105°C; TLC: R_f = 0.75 (20:80 ethyl acetate:hexane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 66.

To a flask containing a suspension of sodium hydride (135 mg, 5.6 mmol) in dimethylformamide (6 mL) was addded di-*tert*-butyl malonate (1.215 g, 5.6 mmol) in dimethylformamide (2 mL) under argon. The reaction mixture was stirred at room temperature for 30 min and then cooled to 0°C. follwed by addition of a solution of **65** (715 mg, 2.57 mmol) in dimethylformamide (8 mL) in one portion. The reaction mixture became clear with a yellow color. The ice bath was removed and the reaction mixture was stirred at room temperature for 1 h. Saturated aqueous ammonium chloride (30 mL) was added and the mixture extracted with ethyl acetate (2 x 30 mL). Organic layers were combined and washed with saturated aqueous sodium chloride (30 mL), dried and concentrated *in vacuo* to give a crude oily material (1.7 g). Recrystalization in hexane (30 mL) gave pure **66** (430 mg). The mother liquor was further purified through silica gel column chromatography, eluting with 5:50:50 ethyl acetate:dichloromethane:hexane, to yield an additional crop of pure **66** (400 mg, total 830 mg, 2.002 mmol, 77.9%). mp 91-92°C; TLC: R_f = 0.80 (20:80 ethyl acetate:hexane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 67.

An aqueous lithium hydroxide solution (1 N, 4.5 mL) was added to a solution of **66** (830 mg, 2.00 mmol) in dioxane (20 mL) at room temperature. After 1.5 h, TLC indicated all starting material had disappeared. 10% aqueous citric acid (10 mL) was added. The precipitate was collected by filtration, rinsed with water and dried *in*

vacuo to give crude **67** (590 mg) which was further purified with flash silica gel column chromatography, eluting with 1:10 methanol:dichloromethane, to yield pure **67** (570 mg, 1.423 mmol, 71.1%). mp 176-178°C; MS: $(M-H)^-$ 399.0
TLC: R_f = 0.47 (ethyl acetate); ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

EXAMPLE 15

This example illustrates the preparation of N-acetyl-L-1,2,3,4-tetrahydro-7-(di-t-butyl malonate methyl)-isoquinoline-3-carboxylic acid, shown schematically in **Figure 28**.

Preparation of 68.

Methanol (35 mL) was cooled to -5°C with an ice-methanol bath followed by slow addition of thionyl chloride (3.5 mL) and L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic, 2.55 g, 14.1 mmol). The ice bath was then removed, the suspension was stirred for 14 h at 30-40°C with a drying tube attached to the reaction flask to protect from moisture. The clear solution was concentrated to dryness to obtain **68** as a crystalline solid (3.11 g, 14.1 mmol, 100%).
TLC: R_f = 0.71 (1:10 methanol:dichloromethane); ^1H NMR (400 MHz, $CDCl_3$ and a drop of CD_3OD) 3.90 ppm (s, 3H, OCH_3): Consistent with proposed structure.

Preparation of 69.

A solution of triethylamine (4.35 mL, 31.2 mmol) and acetic anhydride (1.61 g, 15.6 mmol) was added to a suspension of **68** (3.11 g, 14.1 mmol) in dichloromethane (75 mL) at 0°C. Then ice bath was removed and the reaction mixture was stirred at room temperature for 1 h. The solution was washed sequentially with saturated aqueous sodium chloride, 1 N hydrochloric acid, saturated aqueous sodium bicarbonate and saturated aqueous sodium chloride. The dichloromethane solution was dried and evaporated to give **69** as a colorless oil (3.27 g, quantitative yield). TLC: R_f = 0.50 (50:50:2 ethyl acetate:hexane:methanol); HPLC: R_t = 18.56 min (C18, 1.5 mL/min, 0.1% trifluoroacetic acid-acetonitrile/0.1% trifluoroacetic acid-water, linear gradient from 5% to 95% over 50 min). ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 70.

A mixture of **69** (3.15 g, 13.5 mmol), chloromethyl ether (15 mL, 198.8 mmol) and zinc chloride (6.6 g, 48.4 mmol) was stirred for 63 h at 0°C. The reaction was monitored by analytical HPLC (C18, 1.5 mL/min, 0.1% trifluoroacetic acid-acetonitrile/0.1% trifluoroacetic acid-H_2O, linear gradient from 5% to 95% over 50 min). The reaction appeared to slow after 50 h at 0°C. The reaction mixture was poured into an ice-water mixture (50 mL) and extracted with ethyl acetate (3 x 60 mL). The organic extracts were washed sequentially with saturated sodium bicarbonate (2 x 50 mL), saturated sodium chloride (50 mL), dried and concentrated to a crude oil (3.74 g) which was triturated with ether/hexane to give an oil (3.37 g) which contained **70** as the major component and about 20% of the unreacted starting material **69**. This material was used in the next reaction without further purification. TLC: R_f = 0.50 (50:50:2 ethyl acetate:hexane:methanol); HPLC: R_t = 22.48 min (C18, 1.5 mL/min, 0.1% trifluoroacetic acid-acetonitrile/0.1% trifluoroacetic acid-water, linear gradient from 5% to 95% over 50 min), R_t = 29.23 min (C18, 1.5 mL/min, 0.1% trifluoroacetic acid-acetonitrile/0.1% trifluoroacetic acid-water, linear gradient from 5% to 65% over 35 min). ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 71.

Di-*t*-butyl malonate (346.1 mg, 1.6 mmol) was added to a suspension of sodium hydride (377 mg, 1.57 mmol) in dimethylformamide (2 mL) at room temperature. The reaction mixture was stirred at room temperature for 20 min when all hydrogen evolution had stopped. This solution was added slowly through a syringe under argon to a solution of **70** (270 mg, 0.8 mmol) in dimethylformamide (2 mL) at 0°C. The reaction mixture was stirred at 0°C for 0.5 h and at room temperature for 15 h. Saturated ammonium chloride solution (20 mL) was added and the mixture was extracted with ethyl acetate (3 x 50 mL), dried and concentrated to an oil which was purified by flash silica gel column chromatography, eluting with 1:1 ethyl acetate:hexane. The appropriate fractions were combined and concentrated to obtain relatively pure **71** (184 mg, 0.399 mmol, 50.0%). TLC: R_f = 0.67 (50:50:2 ethyl acetate:hexane:methanol); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 72.

Lithium hydroxide (1 N, 0.8 mL) was added to a solution of **71** (184 mg, 0.399 mmol) in p-dioxane (3 mL) and water (1 mL) at room temperature. The reaction mixture was stirred at room temperature for 1 h when TLC revealed all of the starting material had disappeared. 10% citric acid (3 mL) was added and the reaction mixure was

concentrated *in vacuo*. The residue was redissolved in ethyl acetate (50 mL) and washed sequentially with 10% citric acid and saturated aqueous sodium chloride (30 mL), dried and concentrated to yield crude **72** (285 mg). The solid was redissolved in dichloromethane (50 mL) and filtered through a plug of silanized silica gel (1 cm thick). The filtrate was concentrated to obtain pure **72** (118 mg, 0.264 mmol, 66.2%). mp >75°C (foam); HPLC: R_t = 31.38 min (C18, 1.5 mL/min, 0.1% trifluoroacetic acid-acetonitrile/0.1% trifluoroacetic acid-H_2O linear gradient from 5% to 55% over 30 min); MS: $(M+Na)^+$ 470.2; ^1H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

EXAMPLE 16

This example illustrates the preparation of *tert*-Butyl 7-(carboxymethyloxy)-coumarin-3-carboxylate, shown schematically in **Figure 29**.

Preparation of 73.

A mixture of 2,4-dihydroxy-benzaldehyde (2.070 g, 15 mmol), di-*t*-butyl malonate (3.89 g, 18 mmol) and piperidine (500 mL) in pyridine (5 mL) was heated in an oil bath at 100°C for 3 h. The reaction mixture was concentrated *in vacuo*. The dark colored mixture was dissolved in 5:95 methanol:dichloromethane and shaken with 10% aqueous sodium bisulfite (30 mL). The organic layer was further washed with saturated aqueous sodium chloride, dried and concentrated to give the crude product. Recrystalization in 1:3 ethyl acetate:dichloromethane yielded pure **73** (805 mg). The mother liquor was concentrated and purified further with silica gel column chromatograpy eluting with 1:3 ethyl acetate:dichloromethane. The appropriate fractions were combined, concentrated and recrystallized from dichloromethane/hexane to obtain an additional 550 mg of **73** (total 1.355 g, 5.147 mmol, 34.3%). mp >180°C (became tan and gradually decomposed); TLC: R_f = 0.13 (1:10 ethyl acetate:dichloromethane); ^1H NMR (400 MHz, DMSO-d_6): Consistent with proposed structure.

Preparation of 74a, 74b and 76.

A mixture of **73** (900 mg, 3.42 mmol), methyl bromoacetate (670 mg, 4.38 mmol) and potassium carbonate (670 mg, 4.38 mmol) in dimethylformamide (15 mL) was stirred at room temperature for 1.5 h. The reaction mixture was concentrated to dryness and the residue was suspended in ethyl acetate (50 mL). Insoluble material was removed by filtration and the filtrate was concentrated to a colored oil (1.4 g), redissolved in 1:2 ethyl acetate:dichloromethane (10 mL) and filtered through silica gel with 1:2 ethyl

acetate:dichloromethane (30 mL). The filtrate was concentrated to yield crystalline pale yellow needles of **74a** (1.075 g, 3.196 mmol, 93.4%). TLC: R_f = 0.58 (1:10 ethyl acetate: dichloromethane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Following the procedure set forth above, and replacing methyl bromoacetate with ethyl bromoacetate, the corresponding ethyl ester **74b** (64 mg, 91.5%) was prepared. TLC: R_f = 0.58 (1:10 ethyl acetate: dichloromethane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Replacing methyl bromoacetate with benzyl bromoacetate, and purification by flash silica gel column chromatgraphy, eluting with 1:10 ethyl acetate:dichloromethane, the corresponding benzyl ester **76** (443 mg, 1.083 mmol, 54.1%) was obtained. mp 142-143°C; TLC: R_f = 0.71 (1:10 ethyl acetate: dichloromethane); ^{1}H NMR (400 MHz, $CDCl_3$): Consistent with proposed structure.

Preparation of 75.

Lithium hydroxide (1 N, 4 mL) was added to a solution of **74a** (1.066 g, 3.18 mmol) in p-dioxane (14 mL) at room temperature. The reaction mixture was stirred at room temperature for 3.5 h and TLC revealed most of the starting material disappeared. 10% aqueous citric acid (8 mL) was added and the reaction mixure was concentrated *in vacuo*. The residue was taken up in ethyl acetate (50 mL) and washed sequentially with 10% aqueous citric acid and saturated aqueous sodium chloride (30 mL), dried and concentrated to yield crude **75** (1.0 g). Flash column chromatography eluting with 90:10:0.05 dichloromethane:methanol:acetic acid yielded pure **75** (590 mg, 1.836 mmol, 57.7%). mp >235°C (dec.); TLC: R_f = 0.06 (1:10 methanol:dichloromethane); MS: $(M_2+Na)^+$ 663.3; ^{1}H NMR (400 MHz, DMSO-d_6): Consistent with proposed structure.

Preparation of 77.

A mixture of **76** (440 mg, 1.08 mmol) and 10% palladium-on-carbon catalyst (324 mg) in ethyl acetate (50 mL) containing 1.5% isopropyl alcohol was degassed and hydrogenated in a pressure bottle at 25-30°C for 30 h. The progress of the hydrogenation was monitored with TLC and UV absorption at 350 nm. After the 350 nm peak completely disappeared and λ_{max} became 280 nm, the hydrogen was removed, the catalyst was filtered and the filtrate concentrated *in vacuo* to yield **77** (318 mg, 0.99 mmol, 91%). mp 144-145°C; TLC: R_f = 0.13 (1:10 methanol:dichloromethane); MS: $(M+Na)^+$ 344.9; ^{1}H NMR (400 MHz, DMSO-d_6): Consistent with proposed structure.

EXAMPLE 17

This example illustrates a general procedure for the diethyl phosphorylation of phenolic acids and their subsequent conversion to phosphoric acid esters.

Triethylamine (1 eq, 139 μL) and TBDMSCl (1 eq, 151 mg) are added at room temperature (or at 0°C for large scale reactions) to a solution of 4-hydroxycinnamic acid or 3-(4-hydroxyphenyl)propionic acid (1 mmol, from Aldrich Chemical Co.) in THF (15 mL). After stirring at RT for 30 min, CBr_4 (3 eq, 995 mg) is added and the solution is cooled to 0°C Diethylphosphite (2.0 eq, 258 μl) and triethylamine (3.0 eq, 417 μl) are then added sequentially (keep light out!) and the reaction mixture changes from white to pink in 10-30 min. The reaction is left for overnight stirring with gradual warming to RT, and quenched next day with 5% aq. $NaSO_3/Na_2SO_3$. THF is removed using rotary evaporator, the residue is extracted with diethyl ether, and the organic extracts are discarded. The aqueous layer is acidified with 10% aq. HCl (to pH 2-4) and extracted with ethyl acetate or dichloromethane. The combined organic extracts are dried, concentrated and the residue chromatographed to afford the pure diethylphosphorylated compounds.

BSTFA (10 eq, 2.66 mL) is added at RT to a solution of the diethyl phosphorylated compound (1 eq) which is previously dissolved (or suspended) in dichloromethane (10 mL). After 30 min, the mixture is cooled to 0°C and treated with a dropwise addition of TMSI (8 eq, 1.14 mL). After 1 h at 0°C, and an additional 1 h at RT, the solution is concentrated under reduced pressure. The residue is treated with a mixture of acetonitrile (10 mL), TFA (3 mL) and water (5 mL) for 1 hour at RT. The resulting mixture is concentrated under reduced pressure, and the resulting product is characterized and utilized directly for subsequent reactions.

Using the above procedures, the compounds in **Figure 30** were prepared. All compounds exhibited spectral properties consistent with their assigned structures.

EXAMPLE 18

This example illustrates the preparation of Fmoc-Ser(CH_2(thymin-1-yl))-OH, Fmoc-Ser(CH_2(5-fluorouracil))-OH, Fmoc-Pro(4-trans-OCH_2(thymin-1-yl))-OH, and Fmoc-Pro(4-trans-OCH_2(5-fluorouracil))-OH as Phosphotyrosine Isosteres and Amino Acid Nucleosides.

Preparation of Fmoc-Ser(CH_2(thymin-1-yl))-OH and Fmoc-Ser(CH_2(5-fluorouracil))-OH

These compounds were prepared according to the general scheme in **Figure 31**.

Diisopropylethylamine (50.67 mL, 296.0 mmol) was added with stirring to a suspension of H-Ser-OMe HCl (23.14 g, 148.7 mmol) in THF (250 mL). The mixture was stirred for additional 0.5 h, then cooled to -10°C, and di(t-butyl)carbonate (32.3 g, 148.0 mmol) in THF (15 mL) added with stirring. The cooling bath was removed and the mixture stirred at room temperature for 4 h. Solvent was removed under vacuum, and the residue distributed between diethyl ether (200 mL) and 5% aqueous citric acid (300 mL). The aqueous layer was extracted with diethyl ether (2 x 150 mL), washed with 2.5% aqueous citric acid, dried ($MgSO_4$) and evaporated in vacuum to give the intermediate **90** as viscous oil (Yield 32.4 g (100%). MS $(M+H)^+$ 220.2).

Dimethylsulfide (33.4 mL, 455.0 mmol) was added to compound **90** (12.4 g, 56.9 mmol) in acetonitrile (227 mL) at 0°C. Benzoyl peroxide (54.3 g, 224.0 mmol) was added with stirring in ~15 portions at 0°C over period of 1.5 h. Stirring was continued for another 4 h, and 1 N aqueous sodium hydroxide (200 mL) added along with diethyl ether (200 mL). The aqueous phase was extracted with diethyl ether (2 x 250 mL). The combined organics were washed with 1 N aqueous sodium hydroxide (2 x 250 mL), dried ($MgSO_4$), and evaporated under vacuum. The crude product was purified by silica gel flash chromatography (eluent: hexane:ethyl acetate 9:1, then hexane:ethyl acetate 5:1). Major fraction eluted with the latter solvent was evaporated in vacuum to afford the intermediate **91** as yellowish oil (Yield 11.3 g (71%). MS $(M+H)^+$ 280.2).

Bis(trimethylsilyl)acetamide (24.8 mL, 100.4 mmol) was added to thymine or 5-fluorouracil (50.2 mmol) in dichloromethane (180 mL) under argon atmosphere, and the mixture refluxed for 45 min (until homogenous solution was obtained). The mixture was cooled to room temperature, and the compound **91** (7.0 g, 25.1 mmol) in dichloromethane (40 mL) added, followed by molecular sieves 4 Å (10.4 g). After 10 min, NBS (5.36 g, 30.1 mmol) was added, and the mixture stirred at room temperature for 45 min. Aqueous 15% sodium hydrosulfite (150 mL) and chloroform (200 mL) were added, and the mixture stirred for 10 min and filtered. Aqueous layer was extracted with chloroform (2 x 120 mL). Combined organic layers were washed with brine (3 x 170 mL), dried ($MgSO_4$) and evaporated in vacuum. Crude product was dried at 40°C/0.3 Torr overnight. Diethyl ether (30 mL) was added, and the mixture stirred for 3 h, followed by addition of diethyl ether (15 mL) and hexane (35 mL). The precipitated compounds **92a** or **92b** were filtered, washed with hexane:diethyl ether (1:1, 2 x 20 mL), and dried in vacuum (Compound **92a**: Yield 6.2 g (69%). Compound **92b**: Yield 6.6 g (73%)). An

appropriate intermediate **92a** or **92b** (16.6 mmol) was dissolved in acetonitrile (300 mL), and aqueous 3% sodium carbonate (400 mL) added. The mixture was stirred at room temperature for 16.5 h, and acetonitrile evaporated in vacuum at room temperature. Aqueous solution was acidified to pH 6-6.5 with conc. hydrochloric acid and washed with ethyl acetate (3 x 200 mL). Aqueous layer was further acidified to pH 3.5-4 and extracted with ethyl acetate (4 x 200 mL). Organic layers (from the latter extraction) were dried ($MgSO_4$) and evaporated in vacuum to afford intermediates **93a** or **93b**. (Compound **93a:** Yield 5.2 g (85%). Compound **93b:** Yield 4.2 g (73%)). An appropriate intermediate **93a** or **93b** (12.1 mmol) was dissolved in 10% v/v trifluoroacetic acid in dichloromethane (100 mL), the solution kept at room temperature for 12 h and then evaporated to ~15 mL. Ethanol (40 mL) was added, and products precipitated with diethyl ether (200 mL). The hygroscopic intermediates **94a** or **94b** were washed with diethyl ether, hexane and dried in vacuum. (Compound **94a**: Yield 2.5 g (84%). Compound **94b**: Yield 3.7 g (100%)). An appropriate intermediate **94a** or **94b** (12.1 mmol) and FmocOPfp (5.43 g, 13.4 mmol) were stirred in a mixture of acetone (60 mL) and water (12 mL), while DIEA was added gradually over ~6 h so that the solution was maintained at pH 7.5-8. Resulted homogenous solution was evaporated in vacuum, and the residue distributed between 2.5% aqueous sodium bicarbonate (150 mL) and ethyl acetate (100 mL). Aqueous phase was washed with ethyl acetate (100 mL), cooled to ~5°C, carefully acidified with conc. hydrochloric acid to pH 2.5-3, and extracted with ethyl acetate (3 x 250 mL). Combined organic layers were washed with diluted hydrochloric acid (pH 2.5-3, 2 x 200 mL), dried ($MgSO_4$), and evaported to afford pure compounds **95a** or **95b** as white solids.

Fmoc-Ser(CH_2(thymin-1-yl))-OH 95a:

Yield: 4.44 g (79%); mp 131-135°C; MS:$(M+H)^+$ 466.3; and ^1H NMR in $CDCl_3$ (consistent with proposed structure).

Fmoc-Ser(CH_2(5-fluorouracil))-OH 95b:

Yield: 5.60 g (94%); mp 101-105°C; MS:$(M+H)^+$ 470.2; and ^1H NMR in DMSO-d_6 (consistent with proposed structure).

EXAMPLE 19

This example illustrates the preparation of Fmoc-Pro(4-trans-OCH_2(thymin-1-yl))-OH and Fmoc-Pro(4-trans-OCH_2(5-fluorouracil))-OH, shown schematically in **Figure 32**.

Intermediates Boc-Pro(4-trans-OCH_2(thymin-1-yl))-OBn **99a** and Boc-Pro(4-trans-OCH_2(5-fluorouracil))-OBn **99b** have been prepared from Boc-Pro(4-trans-OCH_2SMe)-OBn **98** (Yield 12.0 g (55% from **97**)) analogously to the synthesis of compounds **92a,b** (see Example 20). Intermediate **99a**: Yield 9.19 g (70%). Intermediate **99b**: Yield 8.62 g (71%). A solution of an appropriate intermediate **99a** or **99b** (16.4 mmol) in methanol (150 mL) was stirred with 10% Pd/C (3.0 g) in the atmosphere of hydrogen for 2 h. The mixture was filtered through Celite and evaporated in vacuum to afford the corresponding intermediate **100a** or **100b** (Intermediate **100a**: Yield 5.9 g (100%). Intermediate **100b**: Yield 6.10 g (99%)). Deprotection of compounds **100a** and **100b** into intermediates **101a,b** with trifluoroacetic acid followed by protection with FmocOPfp into final products **102a,b** were accomplished analogously to the just described preparations of compounds **94** and **95**, respectively (see Example 20). Intermediate **101a**: Yield 4.71 g (90%). Intermediate **101b**: Yield 4.45 g (99%).

Fmoc-Pro(4-trans-OCH_2(thymin-1-yl))-OH 102a:

Yield (from 3.7 g, 13.7 mmol of **101a**): 5.90 g (87%); mp 102-106°C; MS:$(M+H)^+$ 492.1; ^1H NMR in DMSO-d_6 (consistent with proposed structure).

Fmoc-Pro(4-trans-OCH_2(5-fluorouracil))-OH 102b:

Yield (from 0.54 g, 2 mmol of **101b**): 0.55 g (56%); mp 196-199°C; MS:$(M+H)^+$ 496.2; ^1H NMR in DMSO-d_6 (consistent with proposed structure).

EXAMPLE 20

This example illustrates the preparation of Ac-L-Phe($CH_2PO_3Bu_2$)-OH, shown schematically in **Figure 33**.

Preparation of Ac-L-Phe($CH_2PO_3Bu_2$)-OEt, 103

Sodium di-tert-butyl phosphate was generated by the treatment of a solution of di-tert-butyl phosphate (1.9 mL, 10 mL) in anhydrous THF (3 mL) with sodium hydride (95%, 0.202 g, 10 mmol) for 30 min at room temperature. A solution of Ac-L-Phe(CH_2Cl)-OEt (0.566 g, 2 mmol) in anhydrous THF (2 mL) was added and the reaction was stirred overnight. The solvent was concentrated and the residue was taken up in diethyl ether (50 mL) and washed with pH = 4 buffer solution (10 x 2 mL) and saturated aqueous NaCl (10 x 2 mL), dried over Na_2SO_4, and chromatographed on silica (1:1 hexane:acetone as eluent) to give Ac-L-Phe($CH_2PO_3Bu_2$)-OEt (0.25 g, 28%) as an oil. TLC: R_f = 0.34 (1:1 hexane:acetone, visualized by UV and PMA) MS: $(M+1)^+$ 441.

Preparation of Ac-L-Phe($CH_2PO_3Bu_2$)-OH, 104

$LiOH \cdot H_2O$ (34 mg, 0.817 mmol) was added to a solution of Ac-L-Phe($CH_2PO_3Bu_2$)-OEt (0.24 g, 0.545 mmol) in MeOH-H_2O (3:1, 8 mL), and the mixture stirred for 1.5 h at room temperature. After removal of MeOH, 1 N HCl was added to acidify the solution (pH = 2), and it was extracted with ethyl acetate (30 x 4 mL). The combined organic extract was dried over $MgSO_4$, concentrated under the vacuum, yielding 0.167 mg (75%) as film. MS: $(M+1)^+$ 413.

EXAMPLE 21

This example illustrates the preparation of Fmoc-L-Phe(CH_2SO_3Na)OH, shown schematically in **Figure 34**.

The title compound was prepared according to literature procedures (see, Miranda, *et al., J. Med. Chem.* **36:**1681 (1993)).

EXAMPLE 22

This example illustrates the preparation of N^{α}-(9-Fluorenylmethoxycarbonyl)-p-((phosphono)difluoromethyl)-L-phenylalanine (**112**), shown schematically in **Figure 35**.

Preparation of compound 108.

A mixture of α-bromotoluic acid (2.15 g, 0.01mol) and phosphorus tribromide (6mL) was refluxed for 3 h whereupon a yellow solution with some yellow semi-solid was obtained. The reaction mixture was decanted from the residue and rotary evaporated at high vacuum to give a yellow semi-solid. This residue was dissolved in toluene (20 mL) and the solution/suspension was filtered under a blanket of argon. The filtrate was rotary evaporated to dryness at high vacuum to give a white solid. Triethylphosphite (1.9 mL, 0.011 mol) was added dropwise to a solution of the crude acyl bromide (3.1 g) in toluene (50 mL) at 0°C. After addition was completed the mixture was stirred for 40 min at 0°C. The reaction mixture was then rotary evaporated to dryness at high vacuum, the gummy residue redissolved in methylene chloride (40 mL) and then treated with acid washed silica (~20g), filtered after 10 min and the filtrate rotary evaporated to a yellow gum which solidified under vacuum to give the keto phosphonate **108** (1.9 g, 57%). ^1H NMR (400MHz,$CDCl_3$): Consistent with proposed structure.

Preparation of compound 109.

Diethylaminosulfur trifluoride (DAST, 10 mL, 0.08 mol) was added to ice cold keto phosphonate **108** (1.8 g, 0.0054 mol). After stirring overnight at room temperature the mixture was diluted with methylene chloride (40 mL) and added dropwise to an ice cold solution of sodium carbonate (100 mL). The methylene chloride layer was separated, dried (sodium sulfate) and the solvent removed via rotary evaporation to give the desired difluorophosphinate **109** (1 g, 52%). ^{1}H NMR (400MHz,$CDCl_3$): Consistent with proposed structure.

Preparation of compound 110.

Lithium bis(trimethylsilyl)amide (1 M in tetrahydrofuran, 3.6 mL, 0.0036 mol) was added dropwise to a solution of the bromide **109** (1.3 g, 0.0033 mol), the lactone (1.28 g, 0.0036 mol) and hexamethylphosphoramide (7 mL) in tetrahydrofuran (70 mL) at -78°C. After stirring for 45 min at -78°C, ethyl acetate (50 mL) was added and the mixture was washed with water, saturated sodium chloride, dried (over sodium sulfate) and the solvent removed via rotary evaporation. Column chromatography of the crude with 5% ethyl acetate/methylene chloride gave the desired alkylated lactone **110** (1.7 g, 78%). ^{1}H NMR(400 MHz,$CDCl_3$): Consistent with proposed structure. TLC: R_f = 0.5 (10% ethyl acetate/methylene chloride, visualized by PMA)

Preparation of compound 111.

The alkylated lactone **110** (1.4 g, 0.0021 mol) in a small volume of methanol was added to a suspension of palladium (freshly prepared by reduction of palladium dichloride (112 mg) with hydrogen) in ethanol (8 mL) and tetrahydrofuran (4 mL). After vacuum aspiration the reaction flask was filled with hydrogen and the mixture was stirred for 20 h under 50 psi of hydrogen. The reaction mixture was then filtered through celite and the filtrate rotary evaporated to dryness. The residue was then triturated three times with ether and dried under vacuum to give the desired amino acid **111** (800 mg, 100%). ^{1}H NMR(400 MHz,$CDCl_3$): Consistent with proposed structure.

Preparation of compound 112.

Dioxane (5 mL) was added to a solution of the amino acid **111** (800 mg, 2.1 mmol) in water (5 mL) containing sodium bicarbonate (235 mg, 2.8 mmol). The mixture was cooled in an ice-bath and treated with FMOC-N-hydroxysuccinimide (944 mg, 2.8 mmol) in a small amount of dioxane. After stirring for 3 h at room temperature, the reaction mixture was diluted with saturated sodium bicarbonate (30 mL) and extracted with ether. The aqueous layer was acidified to pH 2 with 6 N hydrochloric acid and

extracted with ethyl acetate. The extracts were dried (sodium sulfate) and the solvents were removed to give the FMOC amino acid (1.29 g) as a white solid. Without further purification, the phosphonate in methylene chloride (20 mL) was then treated with bis(trimethylsilyl)trifluoroacetamide (BSTFA, 6.4 mL, 0.0242 mol). After stirring for 1 h at room temperature the mixture was cooled to -20°C and trimethylsilyl iodide (2.5 mL, 0.0176 mol) was added. After stirring for 1 h at -20°C to 0°C, the reaction mixture was stirred for an additional 1 h at room temperature and then concentrated under vacuum to a viscous brown oil. The oil was treated with acetonitrile (4 mL), trifluoroacetic acid (0.5 mL) and water (1 mL) and stirred for 2.5 h at room temperature. The reaction mixture was then concentrated under reduced pressure to give an oil which was redissolved in ethyl acetate (50 mL) and washed with 5% sodium dithionite (acidified) followed by a wash with saturated sodium chloride. The ethyl acetate layer was dried (sodium sulfate) and the solvent was removed under reduced pressure to give the phosphonic acid **112** (1.19 g, 100% yield from **111**). ^{1}H NMR(400 MHz,$CDCl_3$): Consistent with proposed structure.

EXAMPLE 23

This example illustrates the preparation of Fmoc-D-Y(PO_3H_2)-OH (**115a**) and Fmoc-(α-Me)-D,L-Tyr(PO_3H_2)-OH (**115b**), shown schematically in **Figure 36**.

Preparation of Fmoc-O-phospho-D-tyrosine (115a)

t-Butyl(dimethyl)silylchloride (0.38 g, 2.5 mmol) was added to Fmoc-D-Tyr-OH **113a** (1.01 g, 2.5 mmol) in THF (38 mL) under an argon atmosphere, followed by triethylamine (0.35 mL, 2.5 mmol). The mixture was stirred at room temperature for 0.5 h. Carbon tetrabromide (2.5 g, 7.5 mmol) was added, and the mixture cooled to 0°C. Triethylphosphite (0.65 mL, 5 mmol) was added, followed by triethylamine (1.04 mL, 7.5 mmol). The mixture was stirred overnight at room temperature, and 10% aqueous sodium hydrosulfite (~5 mL) was added carefully with cooling in an ice bath (t < 25°C, pH ~4). The mixture was stirred briefly, and 5% aqueous sodium bicarbonate added to pH ~8-8.5, followed by extraction with diethyl ether (2 x 75 mL). The organic layer was backwashed with 2.5% aqueous sodium bicarbonate (90 mL). Combined aqueous layers were carefully acidified to pH ~3.5-4 with concentrated hydrochloric acid and extracted with ethyl acetate (2 x 150 mL). The organic layers were washed with diluted hydrochloric acid (75 mL, to pH ~2), brine (2 x 75 mL), dried and evaporated to afford the intermediate **114a** as a white amorphous solid.

Intermediate Fmoc-D-Y(PO_3Et_2)-OH 114a: Yield: 1.2 g (89%); MS:$(M-H)^-$ 538.1; and ^1H NMR in $CDCl_3$ (consistent with proposed structure).

Bis(trimethylsilyl)trifluoroacetamide (5.26 mL, 19.5 mmol) was added to the compound **114a** (0.98 g, 1.82 mmol) in dichloromethane (20 mL) under argon atmosphere. The mixture was stirred for 1 h at room temperature and then cooled to -20°C, followed by dropwise addition of trimethylsilyliodide (2.0 mL, 14.5 mmol). The mixture was allowed to warm up to room temperature over 2 h, stirred for 1 h, and evaporated in vacuum at room temperature. Acetonitrile (10 mL), water (0.5 mL), and trifluoroacetic acid (0.3 mL) were added, and the mixture stirred for 2 h. Solvents and volatile reagents were removed under vacuum (0.5 Torr, room temperature), and the residue distributed between 5% aqueous sodium hydrosulfite (75 mL) and ethyl acetate (125 mL). The organic layer was separated, and the aqueous layer carefully acidified to pH ~3, followed by extraction with ethyl acetate (2 x 75 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under vacuum to afford the pure product **115a** as an amorphous yellowish solid.

Fmoc-D-Y(PO_3H_2)-OH **115a**: Yield: 0.87 g (99%); MS:$(M-H)^+$ 482.1; ^1H NMR in $CDCl_3$ (consistent with proposed structure).

Preparation of Fmoc-O-phospho-α-methyl-D,L-tyrosine, 115b

The title compound was prepared analogously to the synthesis of compound **115a** from Fmoc-(α-Me)-D,L-Tyr-OH via the intermediate **114b** (Scheme 1). Intermediate Fmoc-(α- Me)-D,L-Tyr(PO_3Et_2)-OH **114b**: Yield (from 1.0 g, 2.4 mmol of **113b**): 0.96 g (73%); MS: $(M+H)^+$ 554.2; ^1H NMR in $CDCl_3$ (consistent with proposed structure).

Fmoc-(α-Me)-D,L-Tyr(PO_3H_2)-OH 115b: Yield (from 0.5 g, 0.9 mmol of **114b**): 0.49 g (100%); MS:$(M+H)^+$ 498.2; and ^1H NMR in $CDCl_3$ (consistent with proposed structure).

EXAMPLE 24

This example illustrates the synthesis of MeNpoc-L-Tyr(PO_3H_2)-OH, shown schematically in **Figure 37**.

L-tyrosine was phosphorylated with pyrophosphoric acid at 80°C for 24 hr and then protected with MeNpoc-Cl using a silylation protocol ((i) BSTFA, (ii) MeNpoc-Cl/DIEA, (iii) AcOH then $NaHCO_3$ then HCl). In total, ~0.5 g of the protected

monomer was prepared and fully characterized (NMR and mass-spectroscopy, elemental analysis).

EXAMPLE 25

This example provides the design and synthesis schemes for a number of conformationally-restricted phosphotyrosine and asparagine derivatives (**Figures 38(a)-38(e)**).

In addition to the targets and synthesis routes shown in **Figures 38a-c**, a number of cyclic versions of natural amino acids can be synthesized as shown in **Figure 38d**. **Figure 38e**, provides the structures of several which have been prepared. Constrained phosphotyrosine analogs are shown schematically in **Figure 39**.

3. Asparagine Isosteres

The presence of an asparagine residue or isosteric replacement in a position "+ 2" to that of tyrosine/phosphotyrosine appears to be of importance for efficient binding of small peptides to SH2 domains of certain proteins. A number of isosteric replacements are shown below. Many of these are available via standard synthetic manipulations on commercially available amino acids. Representative syntheses for some of the isosteres are described below.

EXAMPLE 26

This example illustrates the synthesis of Fmoc-L-Ala(3-(1,3-dithian-2-yl))-OH, shown schematically in **Figure 40**.

The title compound has been designed as an Asn replacement for the construction of ESL and preparation of analogues of SH2-binding phosphopeptides favoring the Asn residue in a position +1 to pY. This amino acid was prepared according to the synthetic scheme above.

EXAMPLE 27

This example illustrates the preparation of 3-acylamino derivatives of Fmoc-alanine, shown schematically in **Figure 41**.

The preparation of N-$^{\alpha}$-Fmoc-3-acylamino-L-alanine (compounds **126a** (R = CH_3) and **126b** (R = H) was carried out using a procedure as described by Knight, *et al.*, *FEBS* **296**:263 (1992).

Bis(trifluoroacetoxy)iodobenzene (10.40 g, 24 mmol) was added to Fmoc-Asn-OH (5.68 g, 16 mmol) and pyridine (3.0 mL) in DMF (40 mL) and water (8 mL). The mixture was stirred at r.t. for 17 h and concentrated (40°C, 0.5 Torr). Residue was triturated with Et_2O (3 x 100 mL), dried (r.t., 0.5 Torr) and stirred at r.t. with Ac_2O/lutidine/THF (20 mL, Applied Biosystems) for preparation of Fmoc-Ala(3-NHAc)-OH **126a**, or AcOCHO (4 mL) for preparation of Fmoc-Ala(3-NHCHO)-OH **126b** in THF (100 mL) for 1 h. Solvent was evaporated, and residue triturated with Et_2O (4 x 40 mL), filtered, and purified by silica gel column chromatography (eluent: $CHCl_3$ - MEOH - AcOH 15: 1: 1) to yield Fmoc-Ala(3-NHAc)-OH (compound **126a** above, 4.2 g, 71%; m.p. 120-128°C; TLC: R_f=0.15 ($CHCl_3$/MEOH/AcOH 15: 1: 1); and MS: $(M+H)^+$ 369.1) and Fmoc-Ala(3-NHCHO)-OH (compound **126b** above, 2.2 g, 39%, m.p. 190-195°C (decomp.), TLC: R_f=0.12 ($CHCl_3$/MEOH/AcOH 15: 1: 1); and MS: $(M+H)^+$ 355.1).

EXAMPLE 28

This example provides the synthesis of Fmoc-L-Ala(3-SO_2NHTrt)-OH, shown schematically in **Figure 42**.

The title compound is an Asn isostere for construction of SH2-binding peptides. In addition, certain L-A(3-SO_2NH_2) derivatives are known to act as inhibitors of the aspartate-synthetase. Notably, no Fmoc-protected derivatives of 3-sulfamoyl-L-alanine have been reported in available literature. This compound can be prepared from L-cystine as shown in the scheme above.

Chlorination of the bis-protected cystine **127** in aqueous AcOH provides the sulfamyl chloride **128**. Further experiments revealed that by contrast to the known reaction with ammonia (Ross, *et al.*, *J. Org. Chem.*, **24**:1372 (1959)) the transformation of the intermediate **128** with tritylamine resulted in β-elimination to give the dehydroalanine **131**.

The desired compound **130** can be obtained from **128** with tritylamine pre-silylated by BSTFA. In this way, ~0.5 g of compound **129** has been synthesized (~45% yield after column chromatography).

EXAMPLE 29

The example provides the synthesis of N-Fmoc-L-δ-methoxynorvaline, shown schematically in **Figure 43**.

Preparation of compound **132**.

Isobutyl chloroformate (1.7 mL, 0.013 mol) was added to a solution of N-t-BOC-L-aspartic acid alpha-t-butyl ester (3.8 g, 0.013 mol) with N-methylmorpholine (1.4 mL, 0.013 mol) in dimethoxyethane (13 mL) at -15°C. After 1 minute of stirring the precipitate was removed by filtration. The filtrate was cooled to -15°C and treated with sodium borohydride (750 mg, 0.0197 mol) in water (7 mL). After the reaction subsided the reaction was quenched with water (350 mL) and extracted with methylene chloride. The extracts were dried (sodium sulfate) and the solvent removed via rotary evaporation to give the desired alcohol **132** (3.0g, 84%). ^{1}H NMR(400 MHz,$CDCl_3$): (Consistent with proposed structure). TLC: R_f=0.55 (10%methanol/ methylene chloride, visualized by PMA)

Preparation of compound **133**.

Chromiun trioxide (4.4 g, 0.044 mol) was added to a solution of pyridine (7.7 g, 8 mL) in chloroform (55 mL). After stirring 15 minutes the alcohol **132** (2.9g, 0.011 mol) in chloroform (11 mL) was added all at once. After 15 minutes of stirring the dark brown liquid was decanted from the tarry residue. The residue was washed with chloroform (2 × 20 mL) and the washings and the decantate were combined and washed with 1N sodium hydroxide, 1N hydrochloric acid, saturated sodium bicarbonate, dried (sodium sulfate) and the solvent was removed via rotary evaporation. The crude material was then filtered through a plug of silica gel with 10% ethyl acetate / methylene chloride to give the desired aldehyde **133** (2.2 g, 73%). ^{1}H NMR(400 MHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **134**.

n-Butyllithium (2.5 M in hexane, 11.2 mL, 0.028 mol) was added to a suspension of methoxymethyl triphenylphosphonium chloride (11.3 g, 0.033 mol) in ether (100 mL) at 0°C. After stirring at 0°C for 10 minutes the aldehyde **133** (2.03 g, 0.0074 mol) in ether (30 mL) was added. The ice bath was removed and the reaction mixture was refluxed for 30 minutes. The resulting mixture was added to water and the ether layer was separated. The aqueous layer was extracted with ether (50 mL) and the two extracts combined, washed with water (2x), dried (sodium sulfate) and the solvent was removed

via rotary evaporation. The resulting crude product was purified by column chromatography with 30% acetone / hexane as eluant to yield the desired enol ether **134** (1.33 g, 60%). ^{1}H NMR (400 MHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **135**.

To a solution of the enol ether **134** (1.33 g, 0.0044 mol) in methanol (30 mL) was added 10% palladium-on-carbon catalyst (0.3 g). The mixture was aspirated and the reaction flask filled with hydrogen and stirred at room temperature for 1 hour. The mixture was filtered and the filtrate was evaporated to dryness under reduced pressure. The crude residue was dissolved in methylene chloride (10 mL) and treated with trifluoroacetic acid (8 mL). After stirring for 1 hour the mixture was evaporated under reduced pressure to give a red oil which was triturated to a pink solid with ether. The crude amino acid was then dissolved in water (5 mL) containing sodium bicarbonate (165 mg, 1.97 mmol) to which was then added dioxane (5 mL). The mixture was cooled to 0°C and treated with Fmoc-N-hydroxysuccinimide (665 mg, 1.97 mmol) in a small amount of dioxane. After stirring for 6 hours at room temperature the mixture was diluted with water (50 mL) and extracted with ethyl acetate. The aqueous layer was acidified with 1N hydrochloric acid to pH 2 and extracted with methylene chloride (2 × 40 mL). The extracts were dried (sodium sulfate) and the solvents were removed via rotary evaporation. The resulting crude material was purified by column chromatography with 15% methanol/methylene chloride, as eluant, to give the desired Fmoc-amino acid **135** (390 mg, 54%). ^{1}H NMR(400 MHz,$CDCl_3$): (Consistent with proposed structure). MS:(M+Na)=392.1. mp 105°C.

EXAMPLE 30

This example illustrates the synthesis of N-Fmoc-L-homoserine methyl ether, shown schematically in **Figure 44**.

Preparation of compound **136**.

Di-t-butyl malonate (2.16 g, 0.01 mol) was added dropwise to a suspension of dry sodium hydride (240 mg, 0.01 mol) in dimethylformamide (30 mL) at 0°C. After stirring at room temperature for 45 minutes, a homogeneous brown solution was obtained to which was added, at ice bath temperature, 2-bromomethoxyethane (1.39g, 0.01 mol). The ice bath was removed and the mixture was stirred at 35°C overnight, poured into saturated ammonium chloride solution and extracted twice with ether. The ether fractions

were dried over magnesium sulfate and the solvent was removed via rotary evaporation to give the crude alkylate **136** (2.45g). ^{1}H NMR (400 mHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **137**.

Trifluoroacetic acid (10 mL) was added to a solution of the crude diester **136** (2.45 g) in methylene chloride (10 mL). After stirring for 1 hour at room temperature the mixture was evaporated to dryness under reduced pressure and the residue was dried under vacuum. The resulting oil was dissolved in acetic acid (30 mL) and the solution was refluxed for 3 hours. The solution was cooled and the acetic acid was removed via rotary evaporation. The crude oily residue was distilled (bulb to bulb) to give the desired acid **137** (790 mg, 67% overall from the malonate). ^{1}H NMR (400MHz,$CDCl_3$): (Consistent with proposed structure). bp = 125°C at 0.1 mmHg.

Preparation of compound **138**.

Triethylamine (881 mg, 8.76 mmol) was added to a solution of the acid **137** (790 mg, 6.7 mmol) in tetrahydrofuran (7 mL). The mixture was cooled to -15°C, treated with pivaloyl chloride (0.9 mL, 7.37 mol), allowed to warm to 0°C and then cooled to -78°C. In another flask the oxazolidinone (2.14 g, 12.06 mmol) in tetrahydrofuran (33 mL) at -78°C was treated with 2.5 M n-butyllithium (5 mL, 12.4 mmol). This solution was then added via syringe to the mixed anhydride at -78°C. The resulting mixture was stirred for 1 hour at -78°C, quenched with saturated ammonium chloride (50 mL), rotary evaporated to remove the tetrahydrofuran and then the aqueous layer was extracted with methylene chloride (3 × 50 mL). The organic extracts were washed with saturated sodium bicarbonate, dried (sodium sulfate) and the solvents removed via rotary evaporation to give the crude product. After column chromatography with 40% ethyl acetate/hexane, the desired material **138** (1.54 g, 83%) was obtained. ^{1}H NMR (400MHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **139**.

To a -78°C solution of 0.5M potassium bis(trimethylsilylamide) (12 mL, 0.006 mol) in tetrahydrofuran (20 mL) was added a precooled solution (-78°C), via canula, of the oxazolidinone **138** (1.5 g, 0.0054 mol) in tetrahydrofuran (20 mL). After stirring 30 minutes at -78°C, a cooled solution (-78°C) of the azide (2.1 g, 0.0068 mol) in tetrahydrofuran (13 mL) was added via syringe. After 5 minutes at -78°C, acetic acid (1.5 mL) was added and the reaction mixture was stirred at room temperature for 1 hour. The mixture was then diluted with methylene chloride (60 mL), washed with saturated

sodium chloride and saturated sodium bicarbonate, dried (sodium sulfate) and the solvents removed via rotary evaporation. After column chromatography of the crude material with 5% methanol/methylene chloride, the desired compound **139** was obtained (1.21 g, 70%). ^{1}H NMR (400MHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **140**.

Lithium hydroxide (195 mg , 0.046 mol) in water (9.25 mL) was added to a solution of the azide **139** (1.21 g, 0.0038 mol) in tetrahydrofuran (26 mL). After stirring for 45 minutes at room temperature the mixture was treated with sodium bicarbonate (692 mg) and the tetrahydrofuran removed by rotary evaporation. The remaining aqueous layer was diluted with water (50 mL) and extracted with methylene chloride (2 × 40 mL). The aqueous layer was then acidified with concentrated hydrochloric acid and extracted with ethyl acetate (4 × 50 mL). The extracts were dried (magnesium sulfate) and the solvent removed via rotary evaporation to give the azido acid. To a solution of the azido acid (547 mg, 3.44 mmol) in methanol (30 mL) was added 10% palladium-on-carbon catalyst (75 mg). The rapidly stirred solution was alternately vacuum aspirated and the reaction flask filled with hydrogen (via a rubber balloon) three times and then stirred under positive hydrogen pressure for 4 hours. The solution was filtered and the filtrate was rotary evaporated to dryness to give the desired amino acid **140** (470 mg, 91% overall). ^{1}H NMR (400MHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **141**.

The amino acid **140**, (470 mg, 3.53 mmol) was dissolved in water (8 mL) containing sodium bicarbonate (300 mg, 3.53 mmol) to which was then added dioxane (8 mL). The mixture was cooled to 0°C and then treated with Fmoc-N-hydroxysuccinimide (1.2 g, 3.53 mmol) in a small amount of dioxane. After stirring for 6 hours at room temperature the mixture was diluted with water (50 mL) and extracted with ethyl acetate. The aqueous was then acidified (1N hydrochloric acid) to pH 2 and extracted with methylene chloride (2 × 40 mL). The extracts were dried (sodium sulfate) and the solvents removed via rotary evaporation. The resulting crude material was then triturated (hexane) to give the desired Fmoc-amino acid **141** (850 mg, 53%). ^{1}H NMR(400 MHz,$CDCl_3$): (Consistent with proposed structure). MS:(M+Na)=377.8. mp 132°C.

EXAMPLE 31

This example illustrates the synthesis N-Fmoc-(2S,3'R,S)-Tetrahydrofuranylglycine, shown schematically in **Figure 45**.

Preparation of compound **142**.

Dimethylsulfoxide (4.5 mL, 65 mmol) in methylene chloride (6 mL) was added dropwise to a solution of oxalyl chloride (1.1 mL, 12.6 mmol) in methylene chloride (75 mL) at -78°C. After stirring for 10 minutes at -60°C the 3-(R,S)-hydroxytetrahydrofuran (1 g, 1.14 mmol) in methylene chloride (10 mL) was added and stirring was continued for 15 minutes at -60°C. Triethylamine (5.72 g, 56.6 mmol) was added to the cooled solution and the dry ice bath was removed. After room temperature was reached, saturated sodium chloride (5 mL) was added and after an additional 10 minutes the two phases were seperated. The methylene chloride layer was dried (sodium sulfate) and the solvent removed via rotary evaporation (very low vacuum) to give the desired ketone (0.979 g). A mixture of the ketone (1 g, 0.012 mol) and the triphenylphosphorane (5.6 g, 0.015 mol) in tetrahydrofuran (64 mL) was stirred at room temperature until the reaction was homogeneous. The mixture was then heated to 55°C and held there for 48 hours. The reaction was then diluted with ethyl acetate (50 mL), washed with water, dried (sodium sulfate) and the solvent removed to give the desired ester **142** (60% overall from the hydroxytetrahydrofuran). ^{1}H NMR (400MHz,$CDCL_3$): (Consistent with the proposed structure). TLC: R_f=0.81 (10% ethyl acetate/methylene chloride, visualized by PMA).

Preparation of compound **143**.

10% palladium-on-carbon catalyst (400 mg) was added to a solution of the ester **142** (1.34 g, 7.30 mmol) in methanol (30 mL). The mixture was aspirated and the reaction flask filled with hydrogen and stirred at room temperature for 1 hour. The mixture was filtered and the filtrate rotary evaporated to dryness. The crude material was then dissolved in 10 mL of methylene chloride and treated with 8 mL of trifluoroacetic acid. After stirring for 1 hour the reaction mixture was rotary evaporated to afford the desired acid **143** (845 mg, 89%) as a light yellow oil. ^{1}H NMR (400MHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **144**.

Triethylamine (1.31 g, 0.013 mol) was added to a solution of the acid **143** (1.3 g, 0.01 mol) in tetrahydrofuran (10 mL). The mixture was cooled to -15°C and treated with

pivaloyl chloride (1.36 mL, 0.011 mol), allowed to warm to 0°C and then recooled to -78°C. In another flask the oxazolidinone (3.2 g, 0.018 mol) in tetrahydrofuran (50 mL) at -78°C was treated with 2.5M n-butyllithium (7.4 mL, 0.0185 mol). This solution was then added added via syringe to the -78°C mixed anhydride solution. The resulting mixture was stirred for 1 hour at -78°C, quenched with saturated ammonium chloride (50 mL), rotary evaporated to remove the tetrahydrofuran and then the aqueous solution was extracted three times with methylene chloride (40 mL). The extracts were washed with saturated sodium bicarbonate, dried (sodium sulfate) and the solvents removed via rotary evaporation to give the crude product. After column chromatography with 40% ethyl acetate/hexane, the desired material **144** (2.02 g, 70%) was obtained. ^{1}H NMR (400MHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **145**.

A precooled solution (-78°C) of the oxazolidinone **144** (2.02 g, 0.007 mol) in tetrahydrofuran (26 mL) was added, via canula, to a -78°C solution (15.6 mL, 0.0078 mol) of 0.5M potassium bis(trimethylsilylamide) in tetrahydrofuran (26 mL). After stirring 30 minutes at -78°C, a cooled solution (-78°C) of the azide (2.7 g, 0.0088 mol) in tetrahydrofuran (17 mL) was added via syringe. After 5 minutes at -78°C, acetic acid (2 mL) was added and the reaction mixture was stirred at room temperature for 1 hour. The mixture was then diluted with methylene chloride (60 mL), washed with saturated sodium chloride and saturated sodium bicarbonate, dried (sodium sulfate) and the solvents removed via rotary evaporation. After column chromatography of the crude material with 5% methanol/methylene chloride, the desired compound **145** was obtained (2 g, 87%). ^{1}H NMR (400MHz,$CDCl_3$): (Consistent with proposed structure).

Preparation of compound **146**.

Lithium hydroxide (300 mg, 0.0070 mol) in water (14 mL) was added to a solution of the azide **145** (2 g, 0.0061 mol) in tetrahydrofuran (40 mL). After stirring for 45 minutes at room temperature the mixture was treated with sodium bicarbonate (1000 mg) and the tetrahydrofuran was removed by rotary evaporation. The remaining aqueous solution was diluted with water (70 mL) and extracted with methylene chloride (2 × 40 mL). The aqueous layer was acidified with concentrated hydrochloric acid and extracted with ethyl acetate (4 × 50 mL). The extracts were dried (magnesium sulfate) and the solvent removed via rotary evaporation to give the azido acid. 10% palladium-on-carbon catalyst (110 mg) was added to a solution of the azido acid (900 mg, 5.27 mmol) in methanol (40 mL). The rapidly stirred solution was alternately vacuum aspirated and the reaction flask filled with hydrogen (via a rubber balloon) three times

and then stirred under positive hydrogen pressure for 4 hours. The solution was then filtered and the filtrate was rotary evaporated to dryness to give the desired amino acid **146** (740 mg, 83% overall). ^{1}H NMR (400 MHz, $CDCl_3$): (Consistent with proposed structure).

Preparation of compound **147**.

The amino acid **146** (740 mg, 5.1 mmol) was dissolved in water (10 mL) containing sodium bicarbonate (428 mg, 5.1 mmol) to which was then added dioxane (10 mL). The mixture was cooled to 0°C and treated with Fmoc-N-hydroxysuccinimide (1.72 g, 5.1 mmol) in a small amount of dioxane. After stirring for 6 hours at room temperature the mixture was diluted with water (50 mL) and extracted with ethyl acetate. The aqueous layer was acidified (1N hydrochloric acid) to pH 2 and extracted with methylene chloride (2 × 40 mL). The extracts were dried (sodium sulfate) and the solvents removed via rotary evaporation. The resulting crude material was then triturated (hexane) to give the desired Fmoc-amino acid **147** (1.5 g, 80%). ^{1}H NMR (400 MHz,$CDCl_3$): (Consistent with proposed structure). MS:(M+Na)=389.8.
mp 135-140°C.

B. Peptide Libraries on Solid Supports

1. Bead-based Libraries

EXAMPLE 32

Construction of a Phosphotyrosine-Containing Encoded Peptide Library

Peptides comprising a phosphopeptide library were synthesized with the general sequence A-pY-X_1-X_2-X_3-S-V, where pY is phosphotyrosine and residues X_1, X_2, and X_3 are taken from the building block set shown below (Cha = cyclohexylalanine; Mso = methionine sulfoxide).

L-Ala	L-Arg	L-Asn	L-Asp	L-Glu	L-Gln
L-Gly	L-His	L-Ile	L-Leu	L-Lys	L-Met
L-Phe	L-Pro	L-Ser	L-Thr	L-Val	L-Tyr
L-Trp	L-Nvl	L-Nle	L-Cha	L-Mso	D-Ile
D-Asn	D-Gln				

17,576 (26^3) different peptides are represented in the library. The ratio of peptide to oligo on each bead is approximately 1000:1. Twenty-six different 3-base codons were employed using the bases 7-deaza A, C, and T. Standard acid labile amino acid side chain protecting groups were employed. Significantly, the phosphate on the phosphotyrosine was not protected during library construction. Model peptide synthesis experiments had indicated efficient, high-yielding peptide synthesis does not require protection of the phosphate moiety.

The initial staining protocol employed three different staining reagents: SH2-GST fusion (the SH2 from Grb2 was employed), rabbit anti-GST IgG, and PE-conjugated goat anti-rabbit. This staining protocol provided only partial resolution of positive and negative control parallel synthesis beads (beads coated with an oligonucleotide and either EpYINQSV or the non-phosphorylated analog of this same sequence). Individual beads from four different gates of the bead population exhibiting binding to the SH2 staining reagents were isolated. The oligonucleotides on these beads were amplified and the soluble DNA was then sequenced. The sequences from the four gates are shown in **Figure 7**. The gate containing the brightest beads in the library (gate 1) contains 0.1% of the library population. Gate 4 contains beads with fluorescence above background levels, but this acquired fluorescence is the weakest of all those beads exhibiting specific binding to the staining reagents. Gate 4 represents 1.2% of the total library population. Gates 2 and 3 have intermediate levels of acquired fluorescence and represent 0.8% and 0.7% of the total bead population, respectively. The average relative fluorescence of the four gates is $1>2>3>4$.

Representative peptides from each of the gates were synthesized and the measured IC_{50}'s are shown in parenthesis.

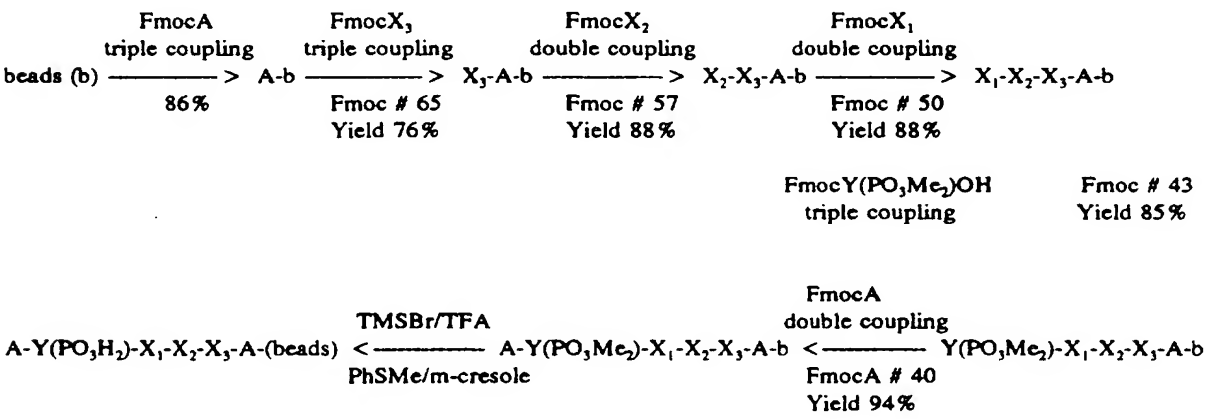
The "positive control" sequence is comprised of residues from the SH2 binding domain of human EGF. Interestingly, one of the library sequences (ApYLNESV) exhibits greater affinity for the SH2 domain than does the positive control sequence (4.5 μM vs. 12 μM).

EXAMPLE 33

Synthesis of O-Phosphotyrosine-Containing Hexapeptide Combinatorial Libraries

Four synthetic O-phosophtyrosine-containing hexapeptide libraries on beads have been prepared for binding activity screening against Src- and Grb2-fusions. In this way, partially degenerate synthetic library A-Y(PO_3H_2)-X_1-X_2-X_3-A (where X_{1-3} = 18 natural amino acids, except for Cys and Trp) has been prepared using the split-synthesis protocol (Furka, *et al.*, *Int. J. Peptide Protein Res.*, **37**:487 (1991); Lam, *et al.*, *Nature*, **354**:82 (1991)), as shown in Scheme 1. The following amino acids have been purchased from Novabiochem and directly used for the libraries construction: FmocAlaOH, FmocAsp(OtBu)OH, FmocGlu(OtBu)OH, FmocPheOH, FmocGlyOH, FmocHis(Trt)OH, FmocIleOH, FmocLys(Boc)OH, FmocValOH, FmocLeuOH, FmocMetOH, FmocAsn(Trt)OH, FmocProOH, FmocGlnOH, FmocArg(Pmc)OH, FmocSer(OtBu)OH, FmocThr(OtBu)OH, and FmocTyr(OtBu)OH.

Scheme 1



X = 18 natural L-amino acids, except for Cys and Trp

Double coupling: (i) Fmoc(Amino Acid) OH/Ho/Bt/HBtU/DIEA;
(ii) Fmoc(Amino Acid) OH/PyBrop/DIEA

Triple coupling: same as above, plus (iii) Fmoc(Amino Acid) OH/EDCI/HOBt/DIEA

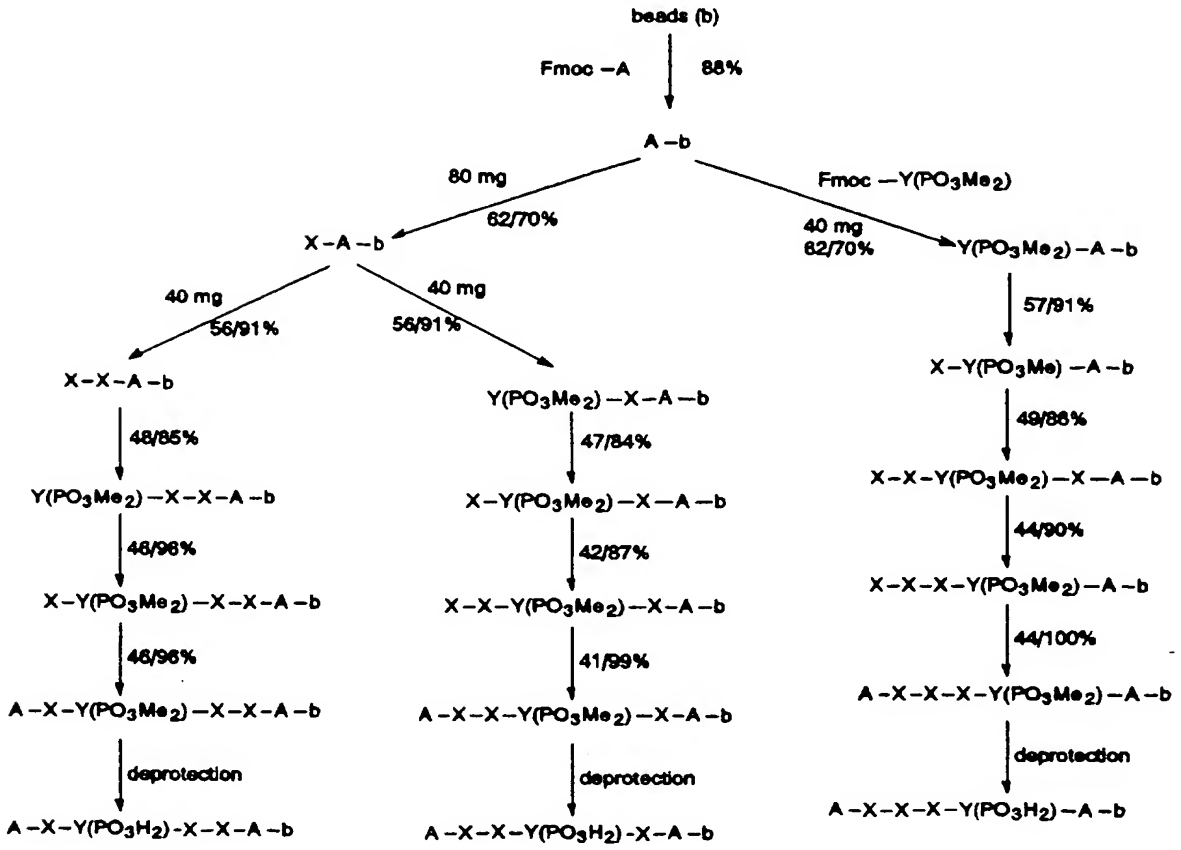
Fmoc numbers are in mmol/g of beads

Monodisperse 10 μm diameter bead material used as a solid support for peptide libraries was a macroporous styrene-divinylbenzene copolymer functionalized with 1,12-diaminodecane linker (Mono A beads, by Pharmacia). O-Phosphotyrosine building block FmocTyr(PO_3Me_2)OH used throughout these studies was prepared as previously reported (see Kitas, *et al.*, *Helv. Chem. Acta.*, **74**:1310 (1991).

A typical coupling cycle involved a vortexing of beads with 1.1 mmol of the Fmoc-protected amino acid in the presence of 1.0 mmol of an appropriate activating reagent and 1.0 mmol of DIEA in a DMF/DCM solution for 40 minutes, followed by washings with DMF, repeated coupling(s), and capping with acetic anhydride in the presence of N-methyl morpholine. The coupling efficiency was controlled by spectrophotometric quantitation of the piperidine-dibenzofulvene adduct ($\epsilon 302 = 7800$ $mol^{-1}cm^{-1}$) formed during the Fmoc group deprotection at the end of each cycle.

These experiments on the couplings of FmocValOH to Monobeads indicated that triple coupling only slightly increased the coupling efficiency as compared to a double coupling (observed values for Fmoc ## of ~86 and 80, respectively). Thus, double coupling protocols were applied in most cases, except when an increase in a coupling was relatively fast (e.g., when single residues were coupled). An introduction of each degenerate residue X included dividing, double/triple couplings, and recombining. (Furka, et al., Id., Lam, et al., Id.). Analogous to the preparation of A-$Y(PO_3H_2)$-X_1-X_2-X_3-A-b, the combinatorial libraries A-X_{-1}-$Y(PO_3H_2)$-X_1-X_2-A-b, A-X_{-2}-X_{-1}-$Y(PO_3H_2)$-X_1-A-b, and A-X_{-3}-X_{-2}-X_{-1}-$Y(PO_3H_2)$-A-b were constructed as shown in Scheme 2 below.

Scheme 2



Numbers given by each arrow represent Fmoc #/Chemical Yield
For designations see Scheme 1
Deprotection: (i) piperidine/DMF
(ii) TMSBr/thioanisole/m-cresole/TFA

Each of 4 libraries is composed of $18^3 = 5832$ peptides. The generic nature of these libraries makes them suitable for screening against all types of SH2 proteins. Deprotected hexapeptide synthetic libraries (40 mg each) are screened vs. Src- and Grb2-glutathione-S-transferase fusions. Thus, the library A-Y-(PO_3H_2)-X_1-X_2-X_3-A-b was treated with Src- and Grb2-glutathione-S-transferase fusions and then subsequently with anti-GST antibody followed by a FITC-conjugated anti-rabbit antibody.

Significantly, a relatively narrow fraction of the library A-Y(PO_3H_2)-X_1-X_2-X_3-A-b appeared to contain peptides of high binding affinity to both Src and Grb2 binding protein SH2 domains. Some of the SH2 active peptides were of relatively short sequence. One percent of the stained library containing most active to Grb2 peptides (with affinity equal

to that of control 11 mer) was separated using fluorescence activated cell sorter and submitted to microsequencing at the Stanford Peptide Sequencing Facility. Microsequencing results are presented in Table 7 below.

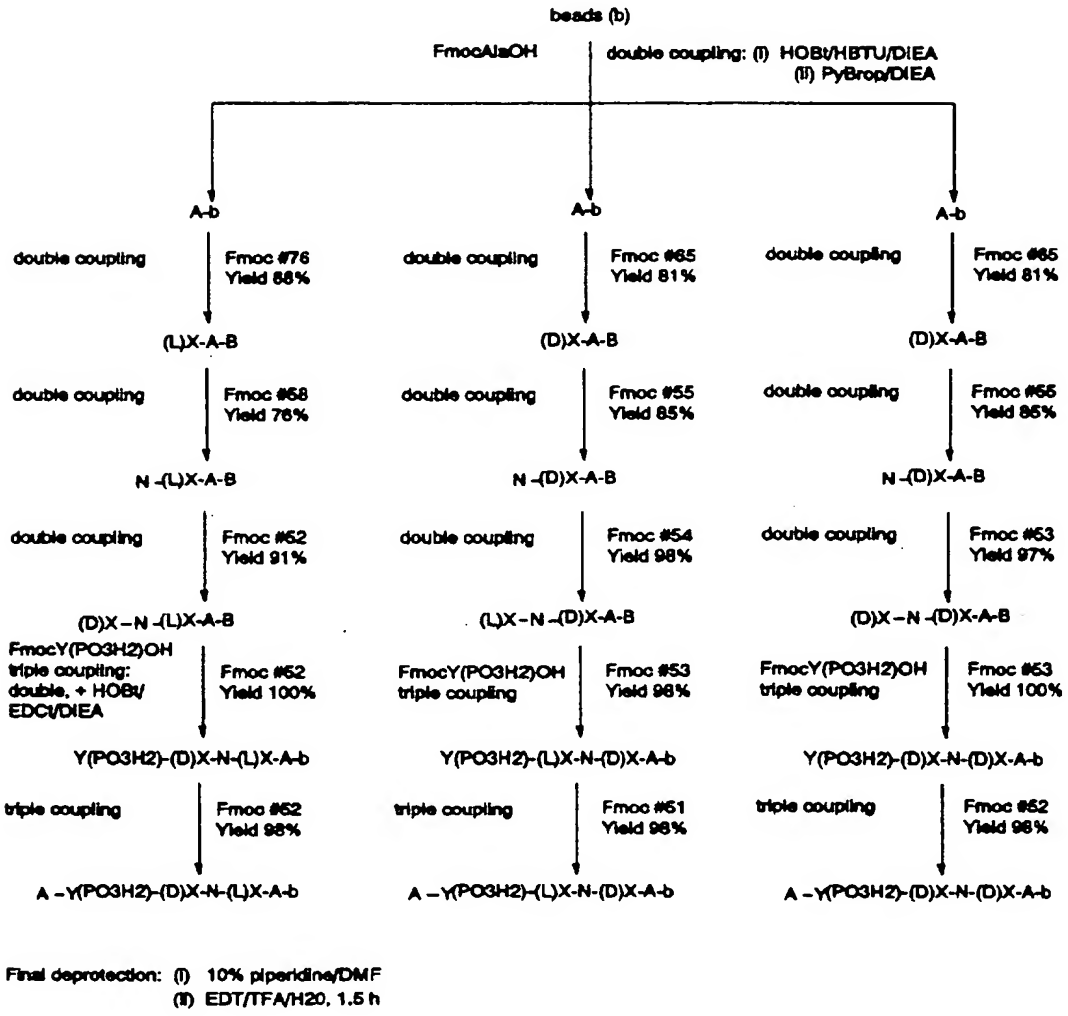
Table 7

Cycle	Amino Acid	Yield in picomoles
1	A	62
2	-	-
3	Q,Y,M,V,F,I,L	1.5-2.0
4	N	39
5	Q,E,M,V,F,I,L	1.5-2.0
6	A	21

Table 10. Microsequencing data of the high affinity library A-Y(PO_3H_2)-X-X-X-A-b. As determined by the Stanford Peptide Sequencing Facility (Edman degradation method).

Degenerate libraries containing D-amino acids were also prepared on beads: A-Y(PO_3H_2)-(D)X-N-(L)X-A-b, A-Y(PO_3H_2)-(L)X-N-(D)X-A, and A-Y(PO_3H_2)-(D)X-N-(D-X-A. These combinatorial libraries were prepared in a manner analogous to the just described procedures using L-amino acids. The D-amino acid building blocks were purchased from Novabiochem, except for Fmoc-D-IleOH purchased from Bachem California. Each library was composed of $18^2 = 324$ peptides.

Scheme 3



The conditions for the final deprotection were determined largely by those required for the removal of Pmc protective group from the residue Arg(Pmc), and included the treatment of bead-supported peptides with 1,2-ethanedithiol and water in TFA at room temperature for 1.5 hour.

EXAMPLE 34

Combinatorial Libraries with Unnatural Amino Acids

Combinatorial libraries containing 34 unnatural amino acids, L-Asp, and L-Glu have been constructed in untagged format using F_2Pmp as a pY isostere (*see* structures in **Figure 46**).

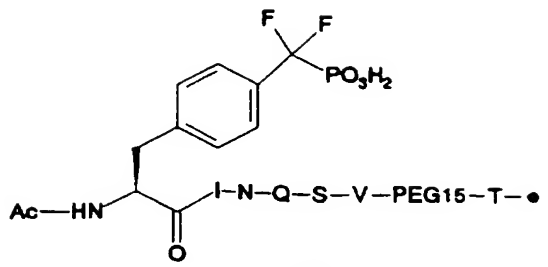
Significantly, the unnatural combinatorial library (third of those in **Figure 46**) was found to contain a detectable amount of SH2-active beads (ca. $< 0.1\%$). This is believed to be the first pY and Asn-free library that contains SH2-binding peptides.

Interestingly, the Asn-containing library displayed a substantial population (up to 40%) of active peptides. This indicated a remarkable tolerance of a position +1 and +2 of the unnatural peptides to pY to various substitutions.

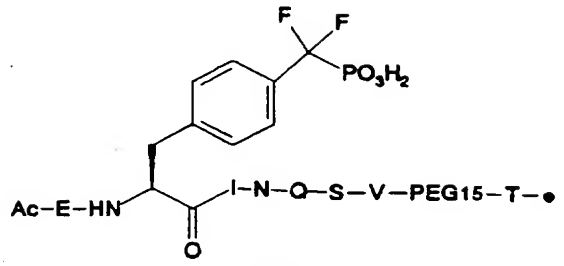
EXAMPLE 35

Construction of Synthetic Libraries with pY Replacements

Earlier results obtained by the present inventors have identified the sequence pY-I-N-Q as a key pharmacophoric unit of the Grb2 SH2-binding peptides. Thus, IC_{50} values for Ac-pY-I-N-Q-NH_2 and its pY-free isostere Ac-F_2Pmp-I-N-Q-NH_2 were determined as $\sim$22 and 91 μM, respectively. To test the ability of the current bioassay MBP-Grb2 SH2-179/Cy3-Ab179 to detect SH2-binding peptides on mono A beads, F_2Pmp-containing peptides on mono A beads were prepared and are represented below.



ACL0050

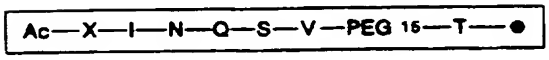


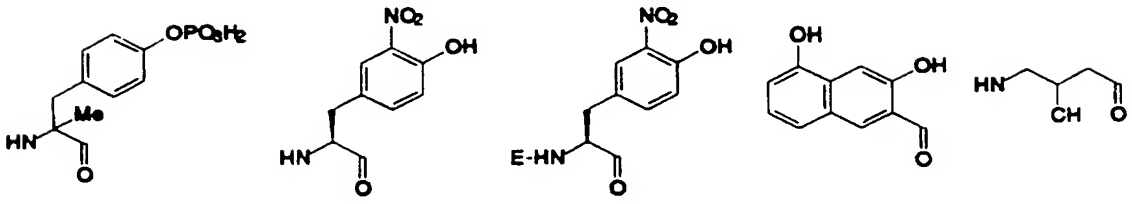
ACL0051

Bioassay experiments revealed a pronounced ability of these polymer-supported peptides to bind to the Grb2 SH2 domain. Notably, in these experiments, peptides on beads displayed affinities toward Grb2 SH2 qualitatively analogous to that of parent pY-based derivatives.

Bead-supported peptides containing the I-N-Q fragment were constructed with other potential pY isosteres (*see* below). Starting peptides Fmoc-I-N(Trt)-Q(Trt)-S(t-Bu)-V-PEG15-T-(mono A beads) were constructed by general methods described herein. Single 1 h HATU coupling protocol was employed to complete the construction of peptides. The final products were capped with Ac_2O/lutidine/NMI and were deprotected by 95% aq. TFA (r.t., 1h). Significantly, during preparations of 3-nitrotyrosine-derived peptides, the nitrophenolate anion (λ_{max} ca. 448 nM) was detected in the 10% piperidine/DMF solution after deprotection of the intermediate Fmoc-Y(3-NO_2)-I-N(Trt)-Q(Trt)-S(t-Bu)-V-PEG15-T-(mono A beads). Evidently, an additional Y(3-NO_2) has been covalently bound to the product during couplings with Fmoc-Y(3-NO_2)-OH, most likely, due to the acylation of the unprotected phenolic hydroxyl. Thus, an additional deprotective procedure has been applied to the preparation of peptides and libraries containing unprotected hydroxyl groups. In this way, the above-mentioned products after aq. TFA deprotection were subjected to the action of sodium bicarbonate in water/MeOH to induce a selective hydrolysis of the undesired esters (saturated aq. $NaHCO_3$-water-MeOH 1:1:2, r.t., 45 min; *see*, Buchi, *et al.*, *J. Am. Chem. Soc.*, **93**:746 (1971)).

Beads-Supported Peptides with Phosphotyrosine Replacements





EXAMPLE 36

Synthesis of Untagged Libraries

Two separate pools of beads with X_1-X_2-X_3-S-V-PEG15-T- and X_1-N-X_3-S-V-PEG15-T- sequences were prepared and split into 36 equal portions. The 36 building blocks (see **Figure 12**) were then coupled manually to generate 36 new libraries (36^3 = 46,656 members each) with the sequence B-X_1-X_2-X_3-S-V-PEG15-T- and another 36 new libraries (36^2 = 1296 members each) with the sequence B-X_1-N-X_3-S-V-PEG15-T-. Of the total 72 libraries, forty were synthesized. Their structures are summarized in **Figures 47(a-d)**.

In general, the coupling yields were high (80-100%) for most of these building blocks, but were slightly lower (60-70%) for α,α-disubstituted and N-substituted α-amino acids. Fmoc numbers that turned out higher than expected (**Figures 47a** and **47d**) can be attributed to errors in dilution or insufficient washing after the coupling reactions.

EXAMPLE 37

Random Untagged Libraries for Grb2-SH2

A set of total random tetrapeptide size libraries have been prepared. In one set of ten libraries, the 36 different monomeric building blocks (see **Figure 12**) were introduced from X_{+1} to X_{+3} positions and one single out of 36 building blocks was introduced individually at X_0 (pY) position. This resulted in ten libraries consisting of pools of $36^3=46656$ members. These libraries are provided in **Figure 47(e)**.

A subset of these libraries with Asn being held constant at X_{+2} position were also prepared. Thus, these libraries have 36 variables each at X_{+1} and X_{+3} position resulting in following ten pools of $36^2=1296$ member libraries provided in **Figure 47(f)**.

EXAMPLE 38

This example illustrates the use of a variety of monomers classes to construct peptides and peptide analogs on beads. The monomer classes include L-amino acids, D-amino acids, aromatic amino acids, basic amino acids, acidic amino acids, backbone-modifying amino acids, neutral H-bonding amino acids, hydrophobic and aliphatic amino acids, lysine derivatives, α,α-disubstituted amino acids, phosphotyrosine and isosteres thereof, and asparagine and isosteres thereof (see **Figures 13(a)-13(i)**).

By systematic preparation of peptide libraries on beads and subsequent screening of the prepared libraries against a target which binds the peptides, an evaluation of each monomers contribution to binding of the target can be made. In this manner, drug discovery is simplified. Thus, a series of bead-based libraries were prepared by the methods described above. These libraries are shown in **Figure 48**.

Figure 48(a) shows seven hexapeptide libraries which were prepared having the N-terminal monomer as phosphotyrosine or one of six phosphotyrosine isosteres. The positions X_1, X_2 and X_3 were individually and randomly selected from a representative group of 36 amino acids which included examples of aromatic amino acids, basic amino acids, acidic amino acids, backbone-modifying amino acids, neutral H-bonding amino acids, and aliphatic amino acids (see **Figure 12**).

Figure 48(b) illustrates three hexapeptide libraries which were prepared having the N-terminal monomer as phosphotyrosine or one of two phosphotyrosine isosteres. The positions X_1, X_2 and X_3 were individually and randomly selected from a group of 19 amino acids and 2 naphthylalanines. In **Figure 48(c)**, two tetrapeptide libraries were

prepared. In these truncated versions, the N-terminal monomer was either phosphotyrosine or 4-(phosphomethyl)phenylalanine and the positions X_1, X_2 and X_3 were individually and randomly selected from a representative group of 36 amino acids (see **Figure 12**).

Figure 48(d) shows five hexapeptide libraries which were prepared having the N-terminal monomer as one of five phosphotyrosine isosteres. The positions X_2 and X_3 were held constant as Asn and Val respectively, and X_1 was randomly selected from the 36 amino acid representative set in **Figure 12**.

Figures 48(e) and **48(f)** show libraries of hexapeptides in which the N-terminal monomers were not phosphotyrosine or a phosphotyrosine isostere, but which were one of the six unnatural amino acids shown. The positions X_1 and X_3 were individually and randomly selected from the above-noted representative group of 36 amino acids. For those libraries in **Figure 48(e)**, X_2 was also randomly selected from the representative set of 36 amino acids. The libraries in **48(f)** were constructed with X_2 held constant as Asn.

The libraries in **Figure 48(g)** are similar to those in **Figure 48(f)**, except that the N-terminal monomer was one of seven phosphotyrosine isosteres.

The libraries in **Figure 48(h)** were constructed to evaluate different linking groups.

Figure 48(i) shows a library of 36 hexapeptides. In this library, the N-terminal monomer was constant (a phosphotyrosine isostere) and only X_1 was varied (one of 36 amino acids in **Figure 12**).

Figure 48(j) shows ten hexapeptide libraries in which the N-terminal monomer is one of ten phosphotyrosine isosteres (including three which are des-amino isosteres). The monomer in the X_1 position is one of the 36 representative amino acids in **Figure 12**. The remaining residues are Asn-Val-Ser-Val.

Figure 48(k) shows seven hexapeptide libraries in which the N-terminal monomer is one of seven phosphotyrosine isosteres. The monomers in the X_1 and X_3 positions are each randomly selected from the 36 representative amino acids in **Figure 12**. The residue in the X_2 position is Asn. Similarly, **Figure 48(l)** shows five tetrapeptide libraries in which the N-terminal monomer is one of five phosphotyrosine isosteres. The monomers in positions X_1, X_2 and X_3 are as described for the libraries in **Figure 48(k)**. Additionally, a PEG30 linker replaces the S-V-PEG15-Thr linker used in the libraries of **Figure 48(k)**.

Figures 48(m)-48(p) show libraries of tripeptides in which the X_2 position is held constant as Asn (or N) and the N-terminal position is a phosphotyrosine isostere. In **Figure 48(m)**, the X_1 position is selected from the 36 amino acid representative set. The libraries shown in **Figures 48(n)-48(p)** were constructed using more fully developed sets

of monomers in the X_1 position. In particular, the monomer classes used at the X_1 position in **Figures 48(n)** and **48(o)** included L-amino acids, D-amino acids, aromatic amino acids, basic amino acids, acidic amino acids, backbone-modifying amino acids, neutral H-bonding amino acids, hydrophobic and aliphatic amino acids and lysine derivatives (see **Figures 13(a)-13(i)**). The monomer classes used at the X_1 position in **Figure 48(p)** included those listed above with the exception of the L-amino acids.

In another series of libraries, the asparagine at position X_2 was substituted with an isosteric replacement. **Figure 48(q)** shows a series of seven hexapeptides in which the N-terminal monomer was phosphotyrosine, and X_1 and X_3 were individually and randomly selected from the representative group of 36 amino acids (see **Figure 12**). The monomer at the X_2 position was either asparagine or one of six isosteric replacements. **Figure 48(r)** shows six similarly constructed libraries in which the phosphotyrosine monomer was replaced with (malonylmethylene)Phe as an isostere of phosphotyrosine.

A series of libraries on beads have also been constructed which are represented by the formula below:

(i) R—AsnI—PEG30—●

(ii) R—X_1(7)—AsnI—PEG30—●

(iii) R—X_1(22)—AsnI—PEG30—●

(iv) pYI—X_1(7)—AsnI—PEG30—●

In the libraries of (i), the group R is any one of the structures **A-J** and **M-SS** in **Figure 49** and AsnI (an asparagine or isostere thereof) is derived from any one of the monomers in **Figure 50**. In the libraries of (ii), the group R is any one of the structures **G-PP** in **Figure 49**, AsnI is any one of the structures in **Figure 50** and X_1 is any one of seven α,α-disubstituted amino acids in **Figure 52**. The libraries of formula (iii) are similar to those of formula (ii) except that R is any one of the structures **A-BB** and X_1 is any one of 22 neutral, H-bonding monomers depicted in **Figure 52**. Similarly, the libraries shown having formula (iv) are similar to those of formula (ii), with the exception of the group R (in (ii)) being replaced by a phosphotyrosine isostere (pYI) selected from the structures provided in **Figure 53**.

C. Peptides

The peptides of the present invention can be prepared by solution phase methods or solid phase methods. General procedures for the methods employed are provided below.

1. Solution Phase Methods

General procedure of coupling reaction and deprotection of Fmoc group

To a mixture of L-amino acids (5 mmol) in 2:1 THF:DMF (15 mL) was added DIEA (1.74 mL, 10 mmol). After stirring for 10 min, the suspension solution was cooled to 0°C and N^{α}-Fmoc-L-amino acid (5 mmol), HOBt.H_2O (0.80 g, 5.25 mmol) and EDCI (1.0 g, 5.25 mmol) were added to the reaction. The reaction mixture was stirred for an additional 20 hrs with gradual warming to room temperature. The solvents were removed in vacuo, and the residue was suspended in ethyl acetate (300 mL), and washed with pH=4 buffer (100 mL x 2), saturated aqueous sodium bicarbonate (100 mL x 2) and saturated aqueous sodium chloride (100 mL), dried over Na_2SO_4, concentrated and purified to give the desired product.

Deprotection of Fmoc group

The Fmoc-protected peptide prepared above (1.0 mmol) was dissolved in a mixture of dimethylformamide (9 mL) and diethylamine (1.0 mL) and the solution was allowed to stir at room temperature for 2 hr. The amine and the solvent were removed in vacuo. The residue was triturated with a mixture of ether (3 mL) and hexane (12 mL), the solid product collected on a filter and washed with a mixture of ether (5 mL)-hexane (5 mL).

Following the above procedures, the following compounds were prepared:

N^{α}-Fmoc-L-Asn(trt)-Gln-$CONH_2$: TLC: R_f=0.64 (4:1 $CHCl_3$/MeOH, visualized by UV and PMA); and MS: $(M+H)^+$ 725.

L-Asn(trt)-Gln-$CONH_2$: TLC: R_f=0.18 (4:1 $CHCl_3$/MeOH, visualized by UV and PMA); and MS: $(M+1)^+$ 504.

N^{α}-Fmoc-L-Ile-Asn(trt)-Gln-$CONH_2$: TLC: R_f=0.67 (4:1 $CHCl_3$/MeOH, visualized by UV and PMA); and MS: $(M+1)^+$ 837.

L-Ile-Asn(trt)-Gln-$CONH_2$: TLC: R_f=0.23 (4:1 $CHCl_3$/MeOH, visualized by UV and PMA); and MS: $(M+1)^+$ 616.

2. Solid Phase Peptide Synthesis

Solid-phase synthesis was carried out starting with an Fmoc-PAL-PS-resin (5g, 1.7 mmol of amino sites/g, 0.11 mmol) and using appropriate N^{α}-Fmoc-amino acids (0.55 mmol each, 5.0 equiv.). The side chain protecting group was Trityl for Asn and Gln. Fmoc removal was with Piperidine-DMF (1:4, 5+20 min), followed by washing with DMF (5 x 30 mL). Couplings were achieved by combining solid N^{α}-Fmoc-amino acids (0.55 mmol), 1-hydroxy-benzotriazole (HOBt) (766 mg, 0.55 mmol) (benzotriazol-1-yl)-tetramethyluranium hexafluorophosphate (HBTU, 1.896g, 0.55 mmol) and N,N-diisopropyl ethylamine (DIEA, 0.174 mL, 1 mmol) in DMF (10 mL) for 1 hr under N_2 at 25°C and then adding this preactivated solution to the peptide-resin. The last coupling of a phosphotyrosine isostere (*e.g.*, N^{α}-Fmoc-L-Tyr($CH_2PO_3H_2$)-OH or N^{α}-Fmoc-L-Tyr(SO_3H)-OH) was carried out by the same protocol, but on small scale (0.034 mmol) by using 2.0 equivalent of amino acids and coupling reagents. After removal of N-terminal Fmoc group, the peptide was N-acetylated with acetic anhydride-lutidine and N-methyl imidazole. The protected peptide-resin was dried and then a TFA-H_2O-ethanedithiol cleavage cocktail was used for deprotection and cleavage from the resin. The cleavage mixture was filtered, and cleaved resin was washed with additional TFA. After concentration, the residue was dissolved in water, washed with hexane-ether, and lyophilized. The crude peptides were purified by RP-HPLC using 4.6 mm x 250 mm column containing 10-μm, 300-A pore-size C-18 packing. Elution from the column was with an acetonitrile/0.1% aqueous trifluoroacetic acid gradient from 0% to 40% acetonitrile linearly over 60 min. All peptides were characterized by ^{1}H-NMR and FAB (fast atom bombardment) mass spectroscopy.

Following the above procedures, a series of tetrapeptides were prepared using four phosphotyrosine isosteres including Ac-Tyr($CH_2PO_3H_2$)-Ile-Asn-Gln-$CONH_2$, Ac-Tyr(SO_3H)-Ile-Asn-Gln-$CONH_2$, Ac-Tyr(CH_2CO_2H)-Ile-Asn-Gln-$CONH_2$ and Ac-Phe($CH_2PO_3H_2$)-Ile-Asn-Gln-$CONH_2$ (structures provided in **Figure 54**).

3. Alternative Synthesis of Linear Peptides on PAL Resin

General Procedure

The following Fmoc-protected amino acids were used in these syntheses: Fmoc-Q(Tmob)-OH, Fmoc-C(Trt)-OH, Fmoc-N(Trt)-OH, Fmoc-I-OH, Fmoc-Y(PO_3H_2)-OH, Fmoc-F_2PmP-OH, Fmoc-E(t-Bu)-OH, Fmoc-A(3-NHAc)-OH and Fmoc-A(3-NHCHO)-OH.

Fmoc-PAL resin (0.26 g, 0.09 mmol, Millipore) was swollen in DMF/DCM (1:1, 3 mL) for 10 min, washed with DMF (3 mL), deprotected with 20% piperidine in DMF (3 mL) for 20 min, and washed again with DMF (3 x 3 mL). The resin was then coupled to an appropriate amino acid (0.50 mmol) with HBTU (0.45 mmol, 0.171 g), HOBt (0.45 mmol, 0.069 g) and DIEA (1.8 mmol, 0.157 mL) in DMF (1.5 mL) for 0.5 - 1 h (until negative Kaiser test) using agitation under nitrogen. Resin was washed with DMF (3 x 3 mL), and deprotection/coupling cycle repeated as above for each of the next amino acid.

After the assembly of desired peptide was completed, the resin was deprotected as above, washed with THF (3 x 3 mL) and acetylated with Ac_2O/lutidine/THF (1 mL, Applied Biosystems) and N-methylimidazole/THF (1 mL, Applied Biosystems) for 10 min. The mixture was then stirred with TFA (2.82 mL), EDT (0.14 mL) and water (0.14 mL) for 1 h at room temperature. The reaction mixture was filtered, the filtrate was concentrated (r.t., 0.5 Torr), crude peptides washed with Et_2O and purified by RV-HPLC (MeCN/0.05% aq. TFA gradient).

Using these procedures the following peptides were prepared: **Ac-F_2PmP-I-N-Q-NH_2; Ac-E-F_2PmP-I-N-Q-NH_2; Ac-Y(PO_3H_2)-I-A(3-NHAc)-Q-NH_2 and Ac-Y(PO_3H_2)-I-A(3-NH-CHO)-Q-NH_2** (structures provided in **Figure 55**). Each of these peptides provided mass spectra consistent with their assigned structures.

4. Preparative Synthesis of Peptides Containing Phosphotyrosine Replacement Building Blocks

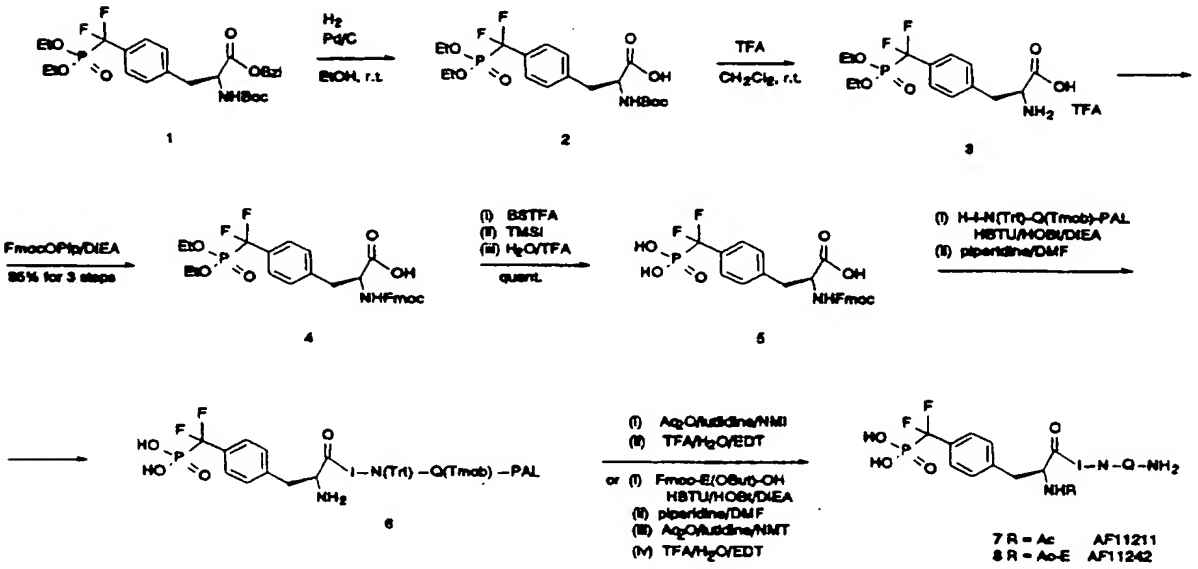
Solid-phase syntheses were carried out on FMOC-PAL-PS-resin using N^{α}-FMOC-amino acids with appropriate side chain protecting groups (trityl for the amide groups of Gln and Asn and t-butyl esters for the carboxyl groups of the phosphotyrosine replacements). The resin (300 mg, 0.102 mmole, Millipore) was swollen in dimethylformamide (DMF, 5 mL) for 10 minutes, washed with DMF (3 x 5 mL), deprotected with 20% piperidine in DMF (5 mL) for 20 minutes and washed again with DMF (5 x 5 mL). It was then coupled with an appropriate amino acid (0.33 mmole, 3.3 eq.) using HATU (0.306 mmole, 3.0 eq.), diisopropylethyl amine (DIEA, 0.92 mmole, 9 eq.) in DMF (1.5 mL) for 1.5 hours (until a negative Kaiser test was obtained) using agitation under nitrogen. Resin was washed with DMF (5 x 5 mL), and the deprotection/coupling cycle was repeated as above for each of the additional amino acids. Finally an appropriate phosphotyrosine replacement building block was introduced in the same manner as mentioned above. After the assembly of desired peptide was completed,

the resin was cleaved from resin by stirring with trifluoroacetic acid (TFA)/water/triisopropyl silane (95 : 2.5 : 2.5) for 1.5 hours at room temperature. The reaction mixture was filtered, washed with TFA and dichloromethane and the filtrate was concentrated (35°C, 0.5 torr). The crude peptides were precipitated and washed in ether and purified by RP-HPLC (acetonitrile/0.1% aqueous TFA gradient). All peptides were characterized by FAB (fast atom bombardment) mass spectrometry. Representative peptides are **Ac-Phe(*p*-CH(S-CH_2-CH_2-SH)HC(CO_2H)$_2$)-Ile-Asn-Gln-NH_2; Ac-Phe(*p*-CH=C-(CO_2H)$_2$)-Ile-Asn-Gln-NH_2; Ac-Phe(*p*-CH_2-HC-(CO_2H)$_2$)-Aze-Asn-Gln-NH_2; Ac-Phe(*p*-CH_2-HC-(CO_2H)$_2$)-Pro-Asn-Gln-NH_2; Cinnamoyl(*p*-CH=C-(CO_2H)$_2$)-Ile-Asn-Gln-NH_2; Cinnamoyl(*p*-CH_2HC-(CO_2H)$_2$)-Ile-Asn-Gln-NH_2; Cinnamoyl(*p*-CH_2HC-(CO_2H)$_2$)-Ile-Asn-NH_2; Cinnamoyl-(*p*-CH_2HC-(CO_2H)$_2$)-Cp-Asn-NH_2; Cinnamoyl(*p*-CH_2HC-(CO_2H)$_2$)-C_b-Asn-NH_2; Cinnamoyl(*p*-CH_2HC-(CO_2H)$_2$)-N_b-Asn-NH_2; Phe(*p*-CH_2-HC-(CO_2H)$_2$)-Ile-Asn-Gln-NH_2; Ac-Phe(*p*-CH_2-HC-(CO_2H)$_2$)-b-Ala--Asn-Gln-NH_2; Ac-Phe(*p*-CH_2-HC-(CO_2H)$_2$)-Aib--Asn-Gln-NH_2; DesaminoPhe(*p*-CH=C-(CO_2H)$_2$)-Ile-Asn-Gln-NH_2; Ac-Phe(*p*-N(CH_2-CO_2H)$_2$)-C_b-Asn-NH_2; 2-Nap(6-(CH(CO_2H)$_2$))-C_b-Asn-NH_2; Ac-Phe(*p*-CH=C-(CO_2H)$_2$)-C_b-Asn-NH_2; Ac-Tiq(6-(CH(CO_2H)$_2$))-C_b-Asn-NH_2; Ac-Phe(*p*-CH_2CH2-$COCH_3$)-C_b-Asn-NH_2; Ac-Phe((*p*-CH_2CF2-COOH))-C_b-Asn-NH_2; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-Glu-Asn-Gln-NH_2; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-homoPhe-Asn-Gln-NH_2; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-Pip-Asn-Gln-NH_2; Ac-Phe(*p*-CH=CCN(CO_2H))-Ile-Asn-Gln-NH_2; Ac-Phe(*p*-CH_2CHCN(CO_2H))-Ile-Asn-Gln-NH_2; Ac-Phe(*p*-CH_2CH(CO_2CH_3)$_2$)-aminospirocyclohexylcarboxylic acid-Asn-$CONH_2$; Ac-Tyr(*p*-CH(CO_2H)$_2$)-aminospirocyclohexylcarboxylic acid-Asn-$CONH_2$; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-Ile-Asn-Gln-$CONH_2$; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-Ile-Asn-$CONH_2$; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-amino spirocyclopentyl carboxylic acid-Asn-Gln-$CONH_2$; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-amino spirocyclopentyl carboxylic acid-Asn-$CONH_2$; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-amino spirocyclohexyl carboxylic acid-Asn-Gln-$CONH_2$; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-amino spirocyclohexyl carboxylic acid-Asn-$CONH_2$; Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-amino norbonanyl carboxylic acid-Asn-Gln-$CONH_2$; and Ac-Phe(*p*-CH_2CH(CO_2H)$_2$)-amino norbonanyl carboxylic acid-Asn-$CONH_2$.**

Synthesis of N-Fmoc Phosphono(difluoromethyl)-L-phenylalanine and Related Phosphonopeptides

Recently, 4-phosphono(difluoromethyl)-L-phenylalanine (F_2Pmp) has received much attention as potent hydrolytically stable O-phosphotyrosine bioisostere for incorporation into SH2-binding peptides. (Burke, *et al., Tetrahedron Lett.*, **34**:4125 (1993); Wrobel, *et al., Tetrahedron Lett.*, **33**:3543 (1993); Burke, *et al., Tetrahedron Lett.*, **35**:551 (1994); Otaka, *et al., Tetrahedron Lett.*, **34**:7039 (1993).) The corresponding N-protected diethylphosphonates (F_2Pmp$(Oet)_2$) have been synthesized both in racemic and enantiomerically pure L-forms. (Burke, *et al., Tetrahedron Lett.*, **34**:4125 (1993); Burke, *et al., Tetrahedron Lett.*, **35**:551 (1994); Otaka, *et al., Tetrahedron Lett.*, **34**:7039, 1993).) The reported use (Otaka, et al., Id.) of the racemic Fmoc-F_2Pmp$(OEt)_2$-OH for preparations of phosphonopeptides requires deprotection of the phosphonate group after construction of peptide sequences is completed. This was achieved under strongly acidic conditions involving the treatment of protected peptides with trimethylsilyl trifluoromethanesulfonate (TMSOTf) and TFA-containing reagents, such as a mixture of TMSOTf/TFA/DMS/EDT. (Otaka, et al., Id.) In certain cases, this may result in complications impeding the synthesis and purification of the target peptides.

Scheme 4



We accomplished the synthesis of Fmoc-L-F_2Pmp $(OEt)_2$-OH (Compound 4 above) from Boc-L-F_2Pmp$(OEt)_2$-OBzl (Compound 1 above) as shown in Scheme 4. In this way, a hydrogenation of 1 over 10% Pd/C in ethanol afforded the acid 2 (Compound 2 above) in quantitative yield. Subsequent deprotection with 10% TFA in CH_2Cl_2 smoothly yielded the compound 3. This simple preparation of the new amino acid 3 (Scheme 4 provides for an easy access to any desirable N-protected form of the building block F_2Pmp). Indeed, the compound 3 was smoothly converted into the Fmoc-derivative 4 (Compound 4 above) with Fmoc-OPfp/DIEA ($[\alpha]D^{25}$ = 42.0°C (c 1.0, $CHCl_3$)). Overall yield of ca. 23% for a new route to the compound 4 employing a known preparation of Boc-L-F_2Pmp$(OEt)_2$-OBzl (Wrobel, et al., Id.) 1 from commercially available Boc-L-Phe-OBzl. Consistent with this, no additional diastereomers have been detected (HPLC and NMR) when the compound 4 was incorporated into peptides Ac-L-F_2Pmp$(OEt)_2$-I-N-Q-NH_2 and Ac-E-F_2Pmp$(OEt)_2$ using standard protocol employing PAL resin.

We have further found that optimal conditions for the deprotection of the diethylphosphonate group in compound 4 included a sequential treatment with bis(trimethylsilyl)trifluoroacetamide (BSTFA; 11 equivalents of the reagent in CH_2Cl_2, r.t., 1 h) and TMSI (8 equivalents, - 20°C to r.t. over 3 h). The crude product thus obtained was dried (0.5 Torr./r.t., 10 h) to remove solvent and excess of reagents and then desilylated with aq. TFA in MeCN (TFA - water - MeCN 1:1.2, r.t., 1 h). This

procedure afforded the desired product 5 (Compound 5 above) in quantitative yield ($[\alpha]D^{25}$ = 7.0°C (c 0.18, DMSO)).

The compound 5 was obtained as a yellowish glass. It can be stored at -18°C for at least 2 months without detectable (HPLC) decomposition. To determine applicability of this new building block toward peptide synthesis in ESL studies, it was used directly for solid phase synthesis of peptides Ac-L-F_2Pmp-I-N-Q-NH_2 7 and Ac-E-F_2Pmp-I-N-Q-NH_2 8. The crude peptides thus obtained were found to be at least 90% pure (HPLC). These compounds were purified by HPLC and characterized by ^1H (COSY), ^{19}F and ^{31}P NMR and FAB mass-spectroscopy.

By contrast to the previously reported observation of side reactions involving free P-OH groups during peptide synthesis with 4-(phosphono)methyl-L-phenylalanine (Shoelson, *et al.*, *Tetrahedron Lett.*, 32:6061 (1991)) no by-products resulted from a coupling of amino and phosphonic groups could be detected in presently reported peptide syntheses using compound 5.

5. Cyclic Peptides

EXAMPLE 39

Synthesis of cyclic phosphopeptides

General Procedure

PAL-supported peptides H-Y(PO_3H_2)-I-N(Trt)-Q(Tmob)-C(Trt)-NH_2 and H-Y(PO_3H_2)-I-N(Trt)-C(Trt)-NH_2 were prepared as described above (except that no acetylation was performed) and then coupled with $BrCH_2COOH$ (0.72 mmol, 0.10 g) and DIC (0.72 mmol, 0.112 mL) in DMF (1.5 mL) for 20 min. Crude linear peptides were cleaved with TFA (2.94 mL) and triethylsilane (0.06 mL) for 1.5 h at r.t. Filtrates were concentrated (r.t., 0.5 Torr), peptides washed with Et_2O (4 x 4 mL) and MeCN (2 x 2 mL), and stirred in aq. NH_4OH (300 mL, pH=8) for 24 h at r.t. Solutions were lyophilized, and crude products purified by RP-HPLC (MeCN/0.05% aq. TFA gradient from 0% to 30% over 35 min).

Using the above procedure, the following peptides (structures in **Figure 56**) were prepared: cyclo(CH_2CO-Y(PO_3H_2)-I-N-Q-C)-NH_2 and cyclo(CH_2CO-Y(PO_3H_2)-I-N-C)-NH_2. Each peptide exhibited spectral features consistent with its assigned structure.

D. Assays

EXAMPLE 40

SH2 Binding and Competition Assays

An iodo-phosphotyrosine peptide based radioligand binding and competition assay was established to determine the binding affinity of peptides to the SH2 domain of Grb2. Microtiter wells were coated with the GST-SH2 fusion protein at 0.5 μg/well (approximately 130 nM). The wells were blocked with bovine serum albumin or any other suitable blocking agent to minimize non-specific binding. Competitor peptides were added at range of concentrations from 0.01 micromolar to 300 micromolar. Iodinated phosphopeptide probe was immediately added, at approximately 30,000 cpm per microliter well. The peptide probe was iodinated using the Bolton-Hunter reagent (3-(p-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester, following the method of Bolton, *Biochem J.* **133**:529 (1973). The wells were incubated at 4°C for 60 minutes, washed with cold buffered saline, and radioactivity counts determined.

In other embodiments, the probe peptide is a fusion of the Kemptide sequences and an SH2-binding phosphopeptide. The fusion peptide is labelled with [^{32}P]ATP and a protein kinase, as described in Kieffer et. al., Anal. Biochem., 215:1-8 (1993).

Results of Using Iodo-Phosphopeptide Probe

Results of an exemplary iodophosphotyrosine competition assay are shown in **Figures 57** and **58**. **Figure 57** shows the competition using the iodopeptide probe FLPVPEpYINQSVP, together with an unlabelled counterpart.

Table 8

Preferred Target	Peptide Sequence	IC_{50} (μM)
Grb2	FLPVPEpYINQSVP-$CONH_2$	+
src	EPQpYEEIPI-$CONH_2$	+
PLCγ	ADEpYLIPQQ-$CONH_2$	-
GAP	SSPpYMAPYD-$CONH_2$	-

Figure 58 shows the specificity of Grb2 specific peptides (open circles) for immobilized Grb2 SH2 domains. Competitor peptides include the peptides described above: a src 12 mer (indicated by closed circles in **Figure 58**), a PLC 9 mer (indicated by open triangles in **Figure 58**), and GAP 9 mer (indicated by closed triangles in **Figure 58**). Peptides known to have lower affinity for the Grb2 SH2 domain, including the polyoma MT peptide (EPQpYEEIPI-$CONH_2$) that binds the src SH2 domain and the EGFR Y992 peptide (ADEpYLIPQQ-$CONH_2$) that binds PLCγ, give right shifted competition curves in the iodo-peptide assay, indicating peptides with lower affinity for the SH2 domain used as target. The results are summarized in Table 8.

Competition assays were also conducted according to the iodophosphopeptide protocol on short conformationally constrained phosphopeptides. The phosphopeptides were conformationally constrained by a disulfide linkage between cysteine residues. Results of the competition assays for these constrained peptides and other SH2 specific peptides are shown in Table 9 below.

Table 9

Peptide Sequence	IC_{50} (μM)
FLPVPEpYINQSVP-NH_2	+
FLPVPEYINQSVP-NH_2	-
EpYINQ-NH_2	+
Ac-EpYINQ-NH_2	+
pYINQ-NH_2	+
Ac-pYINQ-NH_2	+
Ac-pYANA-NH_2	-
Ac-EpYANA-NH_2	-
CpYINQC-NH_2	+
Ac-CpYINQC-NH_2	++
CpYCNQ-NH_2 isomer A	+
CpYCNQ-NH_2 isomer B	+
CEpYCNQ-NH_2	-
Ac-CEpYCNQ-NH_2	+
pYCNC-NH_2	-
Ac-pYCNC-NH_2	+
EpYCNC-NH_2	+
Ac-EpYCNC-NH_2	+
H_2N-EpYINQ-$CONH_2$	++
AcH-N-EpYINQ-IONH_2	+
AcHN-pYINQ-$CONH_2$	+
H_2N-ApYINQA-$CONH_2$	+
AcHN-ApYINQA-$CONH_2$	+
H_2N-ApYINQSV-$CONH_2$	-
H_2N-ApYLNESV-$CONH_2$	+
H_2N-ApYENKSV-$CONH_2$	+
H_2N-ApYLNKSV-$CONH_2$	+

Peptide Sequence	IC_{50} (μM)
H_2N-ApYdNDSV-$CONH_2$	-

Alternative Probes

The iodo-phosphopeptide probe is ideal for determination of the affinity of compounds for the SH2 domain of Grb2. However, a measure of the affinity of test compounds for other SH2 domains that serve as indicators of the specificity or selectivity of the compound is also desirable.

Another alternative probe is an autophosphorylated receptor tyrosine kinase, for example, the EGFR intracellular domain.

EXAMPLE 41

SH2-EGFR Interaction Assay

To demonstrate the specificity of peptide leads for a given SH2 domain, each compound is tested in binding and competition assays analogous to that described in Example 1 for the SH2 domain of Grb2. Leads are tested for their inhibitory potency against other SH2 domains, to characterize their relative specificity in the manner described above for Grb2.

Selectivity Assays

Peptides were designed based on literature sequence reports to use as ligands for GAP, src, and Shc, other SH2 domains that serve as specificity controls. Using EGFR-ICD as probe and three SH2 domains as target, we tested these peptides, as well as several peptides obtained from RPD experiments, with Grb2, Shc, and GAP as targets. The homologous competitions (where a peptide was tested on its presumed cognate SH2 domain) confirmed that each peptide bound specifically, with affinities ranging from 1 to 40 μM. In the heterologous competitions, where peptides were tested for competition on the other SH2 domains, were selectivities of up to several hundred-fold were observed (*see* Table 10). For example, AF-10793 (Ac- C pY I N V P F T C -NH_2), a 9 mer cyclic peptide derived from RPD, (results of a single experiment, not five as in the results shown in Table 4 *supra*) has an IC_{50} for binding to Grb2 of 4.4 nM. The apparent relative affinities for the N-terminal SH2 of GAP and for the SH2 of Shc are 440- and

7500-fold lower, respectively. This substantial selectivity indicates that the compounds of the invention may be used to specifically inhibit Grb2.

Table 10

presumed target	sequence	Grb2	target Shc	nGAP
Grb2	F L P V P E *Y I N Q S V P-NH_2	++	-	+
	F L P V P E Y I N Q S V P-NH_2	-	-	-
	Ac- C *Y I N V P F T C-NH_2	+++	+	+
	E C Y I N V P F T C	+	-	-
	Ac-C *Y I N O C-NH_2	++	-	+
	Ac-C *Y I N V P C-NH_2	++	+	+
GAP	S S N *Y M A P Y D-NH_2	-		+
src	E P Q *Y E E I P I Y L G-NH_2			-

Table 10. Comparison Of IC_{50} values of lead peptides and specificity peptides on homologous a heterologous SH2 domains. Underlining indicates cyclization via a disulfide bond.

EXAMPLE 42

Other Assays

Other biological assays that can be used to demonstrate the activity of the compounds of the present invention are disclosed in ER Wood, *et al, J. Biol. Chem.*, **267**:14138-14114 (1992); G. Zhu, *et al; Proc. Natl. Acad. Sci., USA*, **89**:9559-9563 **12**:5087-5093 (1992); G. Panayatov, *et al, Mol. Cell. Biol.*, **13**:3567-3576 (1993); L. Buday, *et al, Mole. Cell. Biol.*, **13**:1903-1910, (1993); L. Buday, *et al, Cell*, **73**:611-620 (1993) each of which is incorporated herein by reference.

In addition, an instrument designed by Molecular Devices Corp. known as a microphysiometer has been reported to be successfully used for measurement of the effect of agonists and antagonists on various receptors. The basis for this apparatus is the measurement of the alterations in the acidification rate of the extracellular media in response to receptor activation. This instrument can also be used to demonstrate the ability of a compound of the present invention to bind to an SH2 domain (provided that the compound can internalize the cell).

EXAMPLE 43

Identification of Optimal Linker for SH2 Peptide Bead Libraries

Experiments were conducted to identify the optimal linker on Mono A beads using peptides having the known high affinity pY-I-N-Q sequence on different linkers on Mono A beads and comparison of resultant fluorescence levels. The following compounds were included in this study: I-N-Q-J-V-PEG15-Thr-Mono A; I-N-Q-PEG15-Thr-Mono A; I-N-PEG15-Thr-Mono A; I-N-Q-A-A-PEG15-Thr-Mono A; I-N-A-A-A-PEG15-Thr-Mono A; I-N-Q-PEG30-Thr-Mono A; and I-N-PEG30-Thr-Mono A.

The observed fluorescence results indicate that PEG30 provided a better scanning signal than SV-PEG15.

EXAMPLE 44

Deconvolution of the Untagged Peptide-on-Bead Library

In order to identify the preferred residue at position X_{+1} in a tripeptide of sequence PTI-X_{+1}-N, the inventors resynthesized aa library as 36 individual compounds on beads, a so-called "n=1" library. Following staining with the Grb2 detection reagents (as in **Figure 6**), fourteen of the fractions contained strong or very strong binding, with the remainder showing little or no specific binding of the detection reagents (see **Figure 59**). Classifications depended on initially observed fluorescence intensity which, as has been shown in earlier experiments, correlates with affinity (in the range of about 10 μM to 500 μM).

In order to improve the discrimination described *supra* and to identify the best residues, the inventors treated the beads with Grb2 staining reagents under conditions for affinity discrimination. **Figure 60** depicts the fluorescence histogram of a mixture of four deconvolution samples under normal (left) and discriminating (right) conditions. In these experiments the n=1 libraries were mixed in unequal amounts, thus aiding in the identification of the active fraction in mixtures of up to four bead-bound peptides.

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The reference described herein are provided solely for their publication prior to the filing date of this application. Nothing herein is to be construed that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

Such modifications and variations which may be apparent to a person skilled in the art are intended to be within the scope of this invention.

WHAT IS CLAIMED IS:

1. An SH2 binding peptide comprising a core sequence of amino acids Z_7XZ_8X, where each X is a member independently selected from the group consisting of the 20 genetically coded L-amino acids and the stereoisomeric D-amino acids; Z_7 is phosphotyrosine or an isostere thereof; Z_8 is asparagine or an isostere thereof; the amino terminus is acylated; and the peptide is less than 14 amino acids; with the proviso that if Z_7 is phosphotyrosine and Z_8 is asparagine, then the peptide is not $GDGZ_7XZ_8XPLLL$.

2. The peptide of claim 1 comprising the core sequence XZ_7XZ_8X where each X is a member independently selected from the group consisting of the 20 genetically coded L-amino acids and the stereoisomeric D-amino acids.

3. The peptide of claim 2 comprising the core sequence $Z_9Z_7XZ_8X$ where Z_9 is C, E, L, R, S, or V.

4. The peptide of claim 2 comprising the core sequence $Z_9Z_7XZ_8XZ_{10}$ where Z_{10} is C, M, P, S, or W.

5. The peptide of claim 3 comprising the core sequence $Z_9Z_7XZ_8XZ_{10}$ where Z_9 is C, E, L, R, S, or V; Z_{10} is C, M, P, S, or W; and wherein if both Z_9 and Z_{10} are C, then the cysteines optionally are joined by a disulfide bond.

6. The peptide of claim 5 comprising the core sequence $Z_9Z_7Z_{11}Z_8XZ_{10}$ where Z_{11} is E, I, L, M, or N.

7. The peptide of claim 5 comprising the core sequence $Z_9Z_7XZ_8Z_{12}Z_{10}$ where Z_{12} is L, Q, S, V, or W.

8. The peptide of claim 6 comprising the core sequence selected from the group consisting of EpYINQ; pYINQ; pYANA; EpYANA; CpYINQC; CpYINQC wherein the cysteines are joined by a disulfide bond; CpYCNQ; CpYCNQ wherein the cysteines are joined by a disulfide bond; CEpYCNQ wherein the cysteines are joined by a disulfide bond; pYCNC wherein the cysteines are joined by a disulfide bond; EpYCNC; EpYCNC wherein the cysteines are joined by a disulfide bond; CpYINVPFTC; CpYINVPFTC wherein the cysteines are joined by a disulfide bond; ECpINVPFTCM; TECpYLNVPEICA; TECpYLNVPEICA wherein the cysteines are joined by a disulfide bond; CDEVpYVNWSC; CDEVpYVNWSC wherein the cysteines are joined by a disulfide bond; CLSpYMNSPMC; CLSpYMNSPMC wherein the cysteines are joined by a disulfide bond; CpYENLWPYSC; CpYENLWPYSC wherein the cysteines are joined by a disulfide bond; CRERpYENVMC; CRERpYENVMC wherein the cysteines are joined by a disulfide bond; QLpYENWPVLT; CPERpYENVMC; CPERpYENVMC wherein the cysteines are joined by a disulfide bond; QLpYENWPVLT; QERpYENVPGI; and RERYENVWYV.

9. As SH2 binding peptide comprising a sequence of amino acids $Z_1Z_2Z_3Z_4Z_5Z_6X$ where X is a member selected from the group consisting of the 20 genetically coded L-amino acids and the stereoisomeric D-amino acids; Z_1 is C, L, R, S, or V; Z_2 is tyrosine or an isostere thereof; Z_3 is E, I, L, M, or N; Z_4 is asparagine or an

isostere thereof; Z_5 is L, S, V, or W; and Z_6 is M, P, S, or W; and the peptide is less than 14 amino acids.

10. The peptide of claim 9 comprising the core sequence $Z_7Z_1Z_2Z_3Z_4Z_5Z_6X$ where Z_7 is E, G, L, or Q.

11. The peptide of claim 10 comprising the core sequence $Z_7Z_1Z_2Z_3Z_4Z_5Z_6Z_8$ where Z_8 is C, E, F, G, M, P, V, or Y.

12. The peptide of claim 11 comprising the core sequence $Z_7Z_1Z_2Z_3Z_4Z_5Z_6Z_8$ where Z_1 is C or R; Z_3 is E or I; Z_5 is V; Z_6 is P or W; Z_7 is E; and Z_8 is C, E, F, G, M, P, V, or Y.

13. The peptide of claim 12 comprising the core sequence selected from the group consisting of ECINVPFTCMA; TECYLNVPEICA; TECYLNVPEICA wherein the cysteines are joined by a disulfide bond; CDEVYVNWSC; CDEVYVNWSC wherein the cysteines are joined by a disulfide bond; CLSYMNSPMC; CLSYMNSPMC wherein the cysteines are joined by a disulfide bond; CYENLWPYSC; CYENLWPYSC wherein the cysteines are joined by a disulfide bond; CPERYENVMC; CPERYENVMC wherein the cysteines are joined by a disulfide bond; CRERYENVMC; CRERYENVMC wherein the cysteines are joined by a disulfide bond; QLYENWPVLT; QERYENVPGI; and RERYENVWYV.

14. An SH2 binding peptide containing a sequence selected from the group consisting of:

EC*YINVPFTCMA, TEC*YLNVPEICA, CDEV*YVNWSC, CLS*YMNSPMC, C*YENLWP*YSC, CPER*YENVMC, QL*YENWPVLT, QER*YENVPGI, RER*YENVW*YV, LS*YMNSPM, DEL*YENWP, DEV*YVNWS, MEE*YVNWS, QEE*YVNWS, VH*YEN*YMW, *YVNV*YDPL, *YWQNVPES, RSG*YENWPVI, GDEH*YRNSL, EDER*YMNLPW, EER*YMNVMPF, SSER*YENVIF, EEQ*YVNMPWF, QREK*YENWPF, Ac-C*YINVPFTC-NH_2, Ac-C*YINVPFTC-NH_2, EC*YINVPFTC, EC*YINVPFTC, EC*YINVPFTC, EC*YINVPFTC, Ac-CDEV*YVNWSC-NH_2, Ac-CDEV*YVNWSC-NH_2, CDEV*YVNWSC-NH_2, CDEV*YVNWSC-NH_2, QERpYENVPG-NH_2, QER*YENVPG-NH_2, RERpYENVPG-NH_2, RER*YENVPG-NH_2, ECpYINVPFTC-NH_2, ECpYINVPCT-NH_2, ECpYINVCFT-NH_2, CRERpYENVW*YC-NH_2, CRER*YENVW*YC-NH_2, CPERpYENVW*YC-NH_2, CPER*YENVW*YC-NH_2, EGCSSEP*YGVWC, ISDEC*YVNVWDSHDLC, TSSER*YVNDVVWL*YSS, GFDEC*YVNILDSWCVM, DMCEQ*YQNWTAPCILE, GGSCG*YVN*YCELENRL, TPELD*YVNWKSGAA*YC, QREWH*YENWSEIGPCC, KC*YQE*YVNLPCLPX, GLCEQ*YVNLIGPCGH*Y, NHESN*YENWPPSTLTT, and fragments thereof which bind to an SH2 domain, wherein *Y is a member selected from the group consisting of tyrosine, phosphotyrosine, tyrosine isosteres and phosphotyrosine isosteres, wherein underlining denotes a dissulfide link between two Cs, and wherein each N is optionally substituted by an asparagine isostere.

15. The SH2 binding peptide of claim 14, wherein said peptide contains a tripeptide *YXN, wherein X is a member selected from the group consisting of I, C, L,

N, M, E, V, R and Q, and wherein *Y is a member selected from the group consisting of tyrosine, phosphotyrosine and isosteres thereof, and wherein N is optionally an asparagine isostere.

16. The SH2 binding peptide of claim 14 which comprises a cyclic structure.

17. The cyclic SH2 binding peptide of claim 16 wherein said peptide is cyclized by the formation of a disulfide bond between cysteine residues contained or which are introduced therein.

18. The cyclic SH2 binding peptide of claim 16 wherein said peptide is modified by the introduction of one or two residues which permit formation of a cyclic structure.

19. The cyclic SH2 binding peptide of claim 18, wherein said residues are selected from the group consisting of cysteine, homocysteine, penicillamine, and PmP.

20. The cyclic SH2 binding peptide of claim 16, wherein said cyclic structure includes the *YXN group, and X is selected from I, E, and V.

21. The cyclic peptide of claim 20, said peptide having from 6 to 11 amino acids.

22. The cyclic peptide of claim 20, said peptide having from 7 to 9 amino acids.

23. The cyclic peptide of claim 15, wherein said peptide has been phosphorylated.

24. An SH2 binding peptide comprising the sequence *YWQNVPES or fragments thereof, wherein *Y is a member selected from the group consisting of tyrosine, phosphotyrosine, tyrosine isosteres and phosphotyrosine isosteres.

25. An SH2 binding peptide containing the sequence AENAE*YLRVAPQG, wherein *Y is a member selected from the group consisting of tyrosine, phosphotyrosine, tyrosine isosteres and phosphotyrosine isosteres.

26. A pharmaceutical composition comprising a pharmaceutically acceptable amount of a peptide according to claim 1 in a pharmaceutically acceptable carrier.

27. A method of treating a disease associated with aberrant cell growth, differentiation or regulation which is associated with defects in receptor tyrosine kinase pathways comprising administering to a patient having said disease, a peptide according to claim 1 in an amount sufficient to partially block or inhibit a cellular signal transduction pathway.

28. The method of claim 27 wherein said disease is selected from the group consisting of cancer, developmental and differentiation diseases, and insulin-resistant (or non-insulin dependent) diabetes.

29. A library useful for identifying ligands capable of binding to SH2 domains, said library comprising:

a plurality of members, each member comprising a solid support covalently coupled to a carboxyl terminus of a peptide, wherein said peptide comprises a sequence of amino acids selected from the group consisting of:

*Y-X-X-X-X;

X-*Y-X-X-X;

X-X-*Y-X-X; and

X-X-X-*Y-X;

wherein,

*Y is a member selected from the group consisting of tyrosine, phosphotyrosine and isosteres thereof; each X is a member independently selected from the group consisting of the 20 genetically coded L-amino acids and the stereoisomeric D-amino acids; and at least one member is capable of binding to an SH2 domain with a binding affinity of less than about 1×10^{-4}M.

30. A library useful for identifying ligands capable of binding to SH2 domains, said library comprising:

a plurality of members, each member comprising a solid support covalently coupled to a carboxyl terminus of a peptide, wherein said peptide comprises a sequence of amino acids selected from the group consisting of:

*Y-A-X-X-X;

*Y-X-A-X-X; and

*Y-X-X-A-X;

wherein,

*Y is a member selected from the group consisting of tyrosine, phosphotyrosine and isosteres thereof; each X is a member independently selected from the group consisting of the 20 genetically coded L-amino acids and the stereoisomeric D-amino acids; and at least one member is capable of binding to an SH2 domain with a binding affinity of less than about 1×10^{-4}M.

31. A library useful for identifying ligands capable of binding to SH2 domains, and library comprising:

a plurality of members, each member comprising a solid support covalently coupled to a carboxyl terminus of a peptide, wherein said peptide comprises a sequence of amino acids selected from the group consisting of:

*Y-dX-X-X-X;

*Y-X-dX-X-X;

*Y-X-X-dX-X; and

*Y-dX-X-dX-X;

wherein,

*Y is a member selected from the group consisting of tyrosine, phosphotyrosine and isosteres thereof; each dX is a member independently selected from the group consisting of the 20 D-amino acids which are stereoisomeric to the 20 genetically coded-L-amino acids; each X is a member independently selected from the group consisting of the 20 genetically coded L-amino acids and the stereoisomeric D-amino acids; and at least one member is capable of binding to an SH2 domain with a binding affinity of less than about 1×10^{-4}M.

32. A library useful for identifying SH2 domain ligands which lack tyrosine or phosphotyrosine, but which bind to a SH2 domain, said library comprising:

a plurality of members, each member comprising a solid to which is covalently bound a peptide having the following general sequence:

X - X - X - X - X - X , wherein X can be any of the naturally coded amino acids except for tyrosine.

33. The library of claim 29, wherein said SH2 domain is an SH2 domain of a protein selected from the group consisting of src, abl, syk, PTP1C, PLC, GAP, SHPTP2, Vav, p85, p13K, c-Crk, SHC, Nck, ISGF3, Sem-5, Grb2, and ZAP 70.

34. The library of claim 29, wherein said member further comprises an oligonucleotide tag which encodes for said sequence of amino acids.

35. A method for identifying an SH2 binding peptide comprising contacting the respective members of a library according to claim 29, with an SH2 domain containing

protein or SH2 domain fragment, and identifying SH2 binding peptides on the basis of those members which bind to the SH2 domain containing protein or SH2 domain fragment with a binding affinity of less than 1×10^{-4} M.

36. A method for producing SH2 peptides having enhanced properties comprising producing a modified SH2 domain binding peptide, wherein the unmodified SH2 peptide contains the sequence "YXN" wherein X is any amino acid, wherein the modification comprises substitution of the asparagine in the "YXN" group with an asparagine isostere.

37. A library useful for identifying ligands capable of binding to SH2 domains, said library comprising:

a plurality of members, each member comprising a solid support covalently coupled to a carboxyl terminus of a peptide, wherein said peptide comprises a sequence of amino acids selected from the group consisting of:

*Y-X-X-X-X;

X-*Y-X-X-X;

X-X-*Y-X-X; and

X-X-X-*Y-X;

wherein,

*Y is a member selected from the group consisting of tyrosine, phosphotyrosine and isosteres thereof; each X is a member independently selected from the group consisting of aromatic amino acids, basic amino acids, acidic amino acids, backbone modifying amino acids, neutral hydrogen-bonding amino acids, hydrophobic and aliphatic amino acids,

lysine derivatives, the 20 genetically coded L-amino acids and the stereoisomeric D-amino acids; and at least one member is capable of binding to an SH2 domain with a binding affinity of less than about 1 x 10^{-4}M.

FIGURE 1

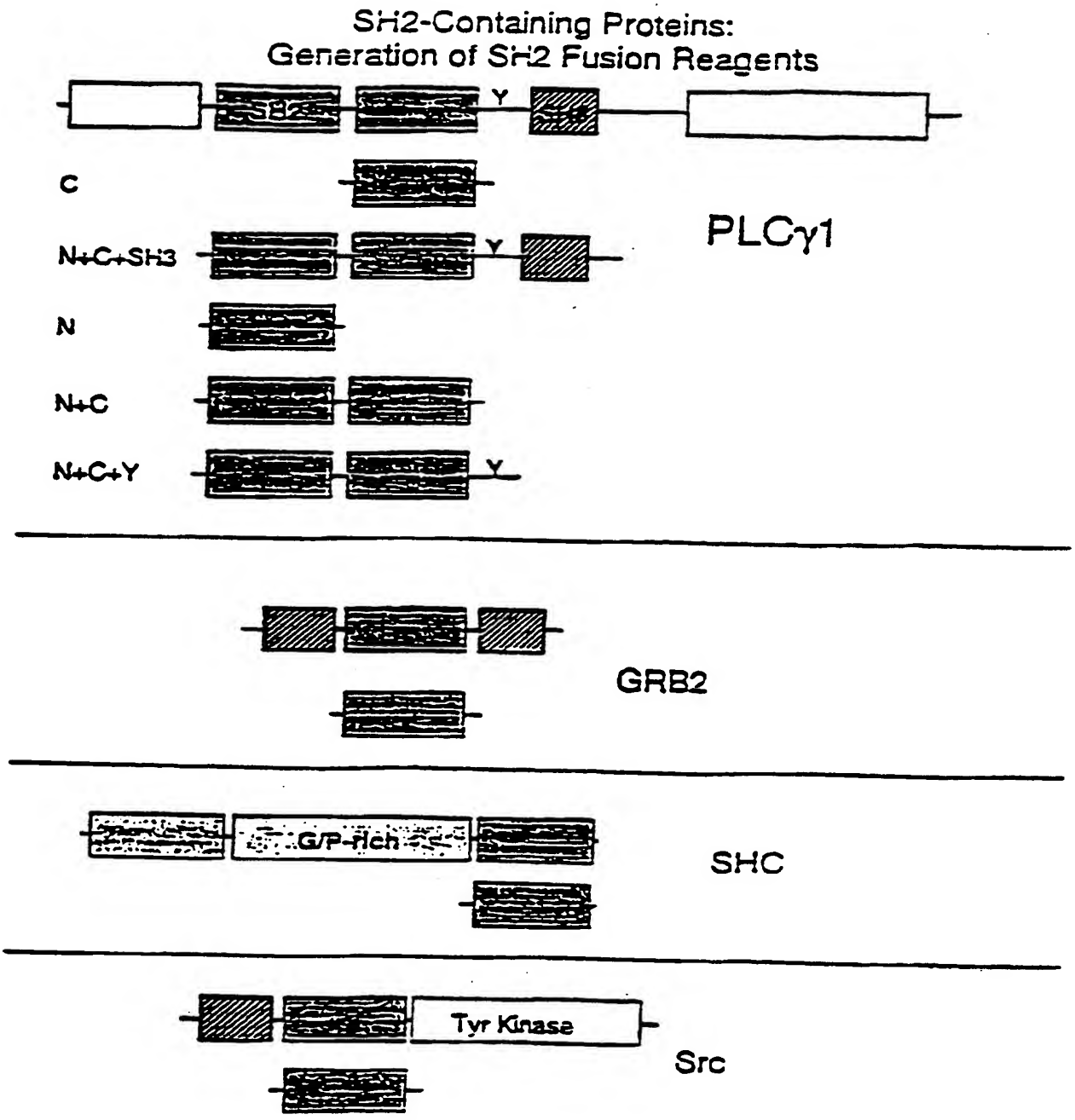


Figure 2. Schematic map of the MBP- and GST-fusion vectors for expression of SH2 domains

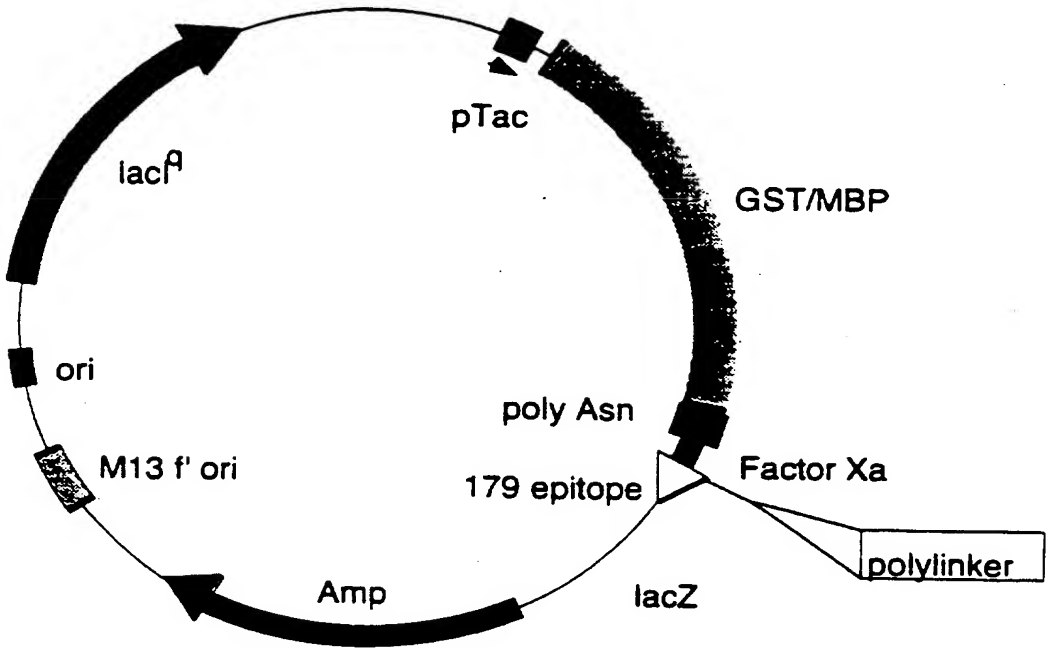
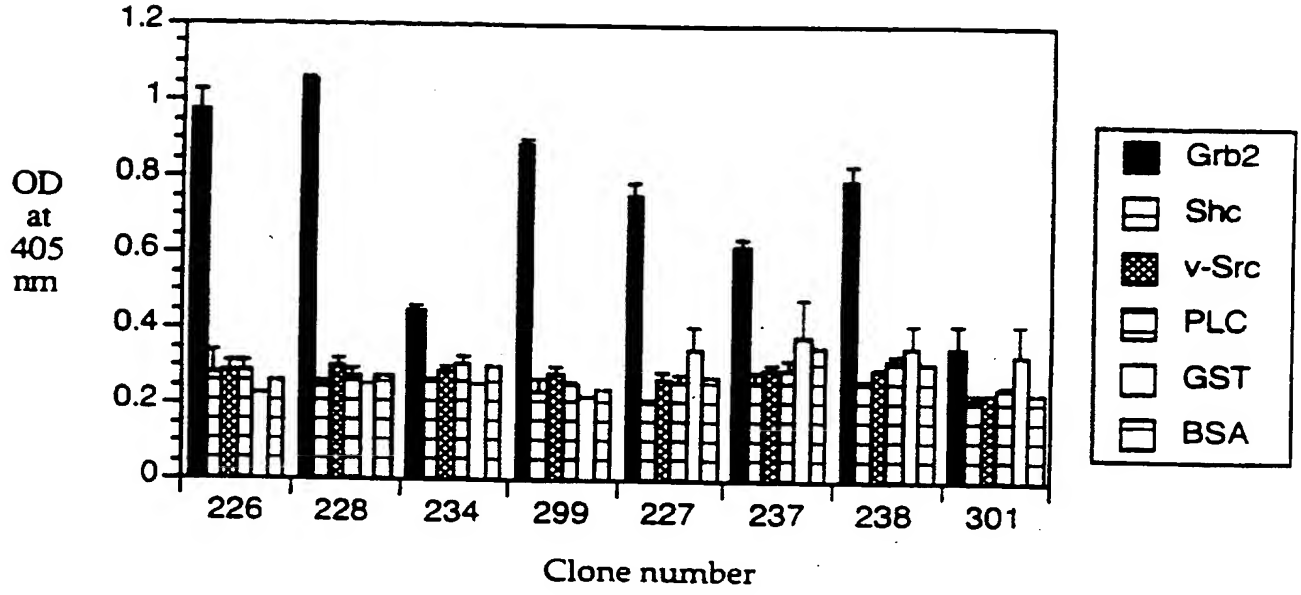


FIGURE 3



Phage ELISA: target specificity of peptides on Grb2-positive phage. Phage were tested on 3 alternative SH2 domain targets, in the standard assay format.

FIGURE 4

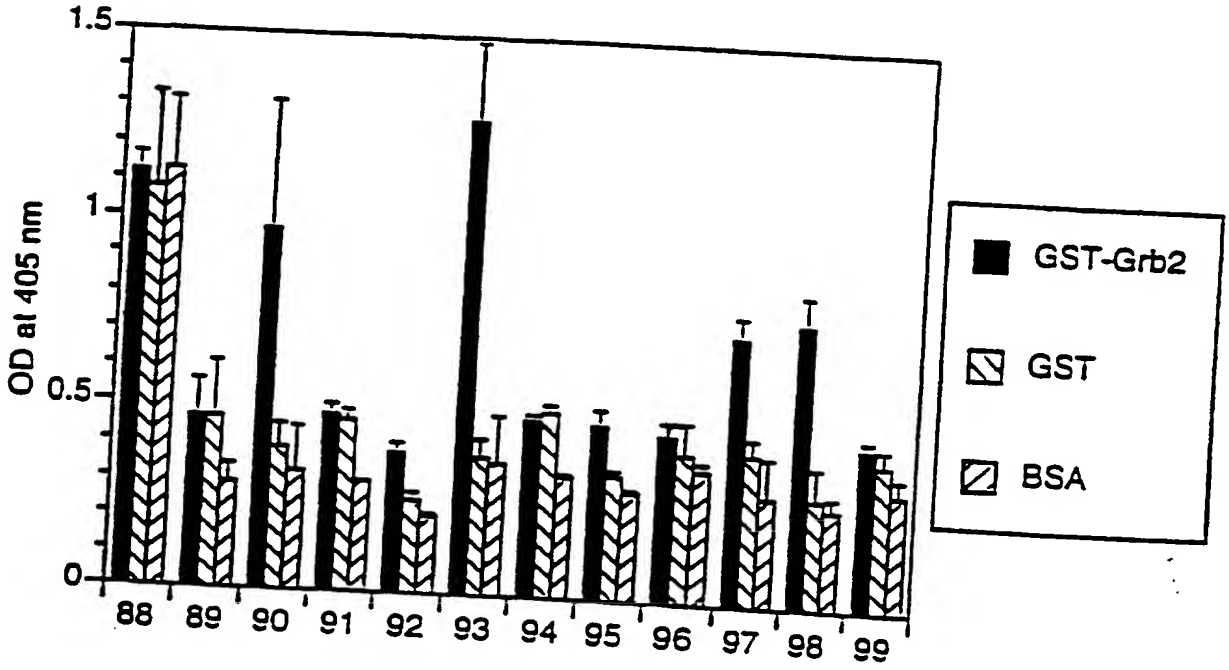


FIGURE 5

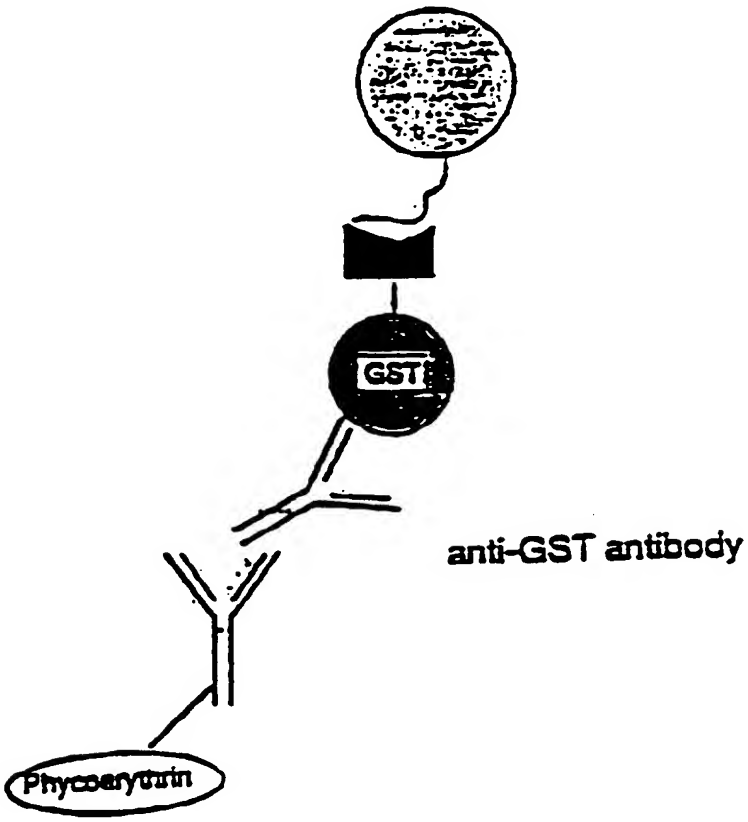


FIGURE 6

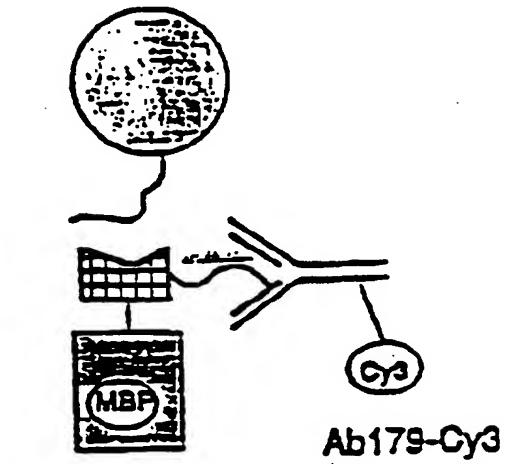


FIGURE 7

A	pY	X_1	X_2	X_3	
		Positive Control			
		I	N	Q	(12 uM)
		Gate 1			
		Q	N	Nvl	
		dQ	N	D	(560 uM)
		E	N	P	
		Cha	N	N	
		A	N	M	
		Nle	N	S	
		I	N	S	
		R	N	Mso	
		Mso	N	?	
		V	N	E	
		L	N	K	(72 uM)
		E	N	K	(51 uM)
		L	N	E	(4.5 uM)
		Nvl	N	A	(30-70 uM)
		R	N	A	
		?	N	E	
		Mso	N	L	
		N	N	I	
		E	N	A	
		Nvl	N	Y	
		E	N	A	
		Gate 2			
		Nle	N	T	
		Nle	N	T	
		A	N	F	(80 uM)
		L	N	A	
		Gate 3			
=		V	Q	Y	
		H	N	K	(400 uM)
		dN	N	V	
		Nvl	N	G	(12 uM)
		?	N	dN	
		N	N	N	
		S	N	Cha	
		A	N	Q	
		Gate 4			
		N	N	Nle	
=		Q	V	Y	
=		Q	S	F	
=		Q	T	Q	(>540 uM)
=		A	dN	M	
		Mso	N	Nle	
=		E	dN	Q	(540 uM)
		Y	N	Y	
=		Q	L	dQ	

FIGURE 8

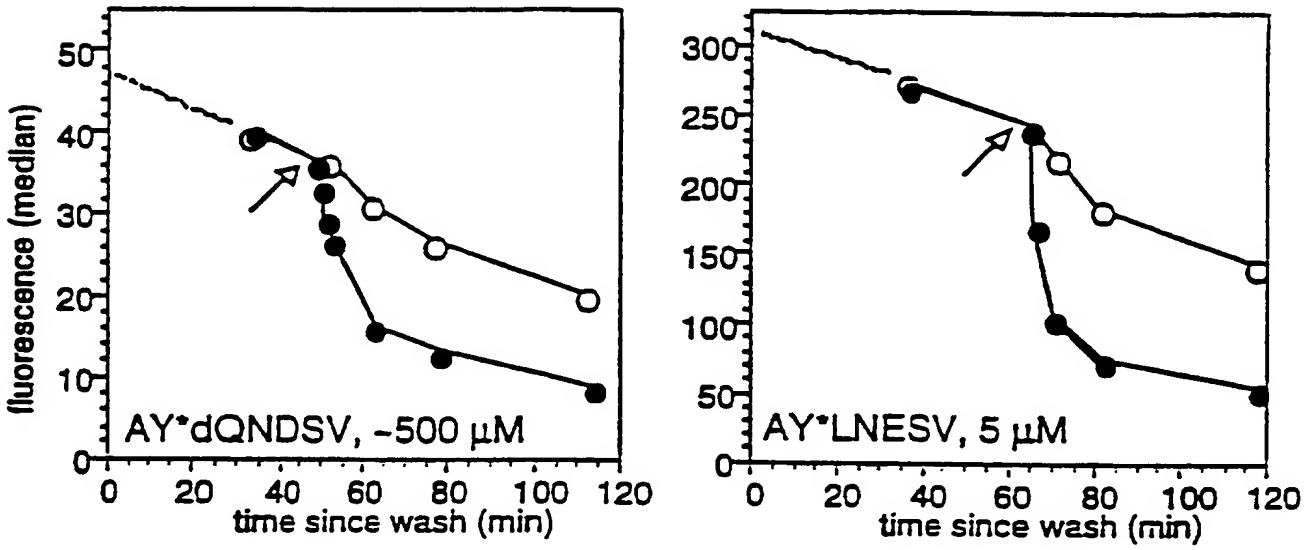


FIGURE 9

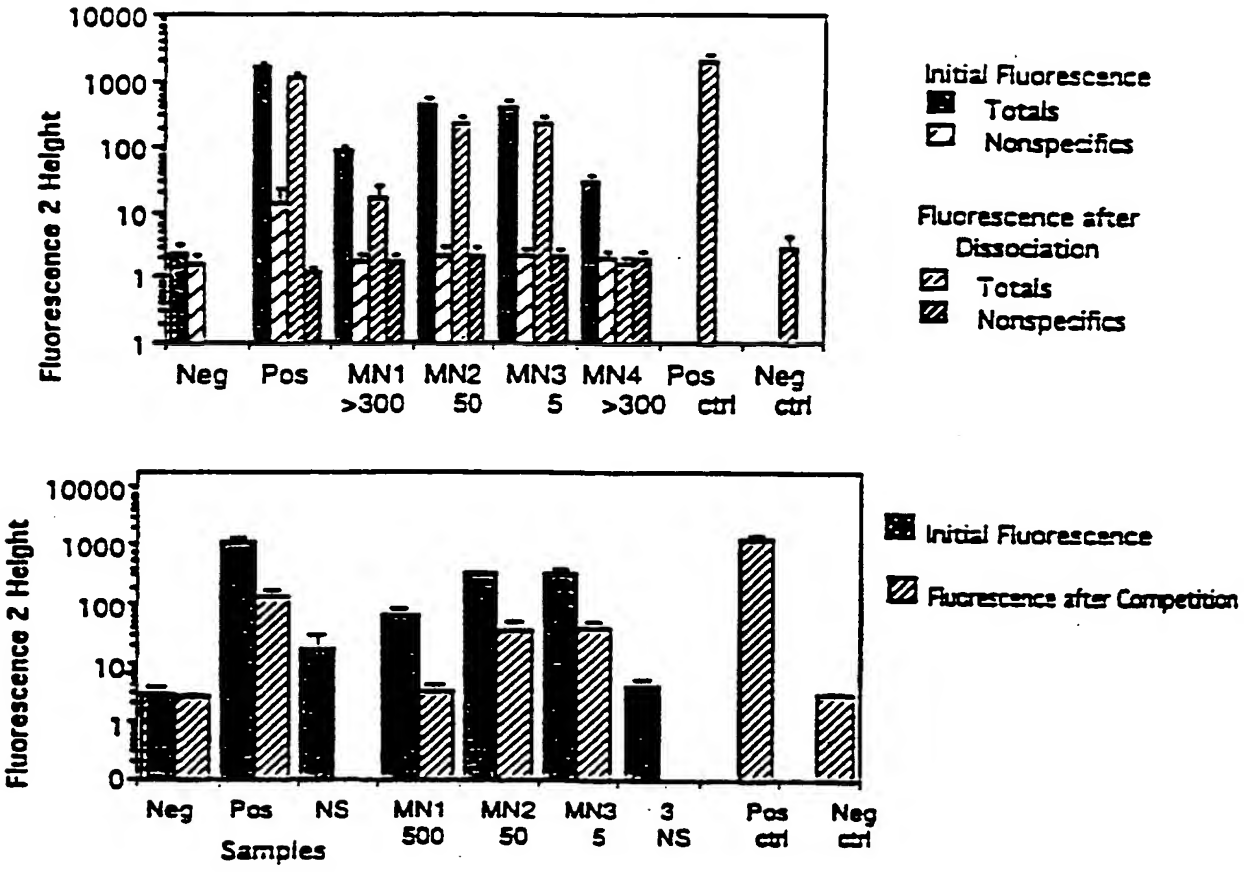

10000
1000
100
10
1
1
Fluorescence 2 Height
Neg
Pos
MN1 >300
MN2 50
MN3 5
MN4 >300
Pos ctrl
Neg ctrl
Initial Fluorescence
Totals
Nonspecifics
Fluorescence after Dissociation
Totals
Nonspecifics
10000
1000
100
10
1
0
Fluorescence 2 Height
Neg
Pos
NS
MN1 500
MN2 50
MN3 5
3 NS
Pos ctrl
Neg ctrl
Samples
Initial Fluorescence
Fluorescence after Competition

FIGURE 10(a)

TYROSINE / PHOSPHOTYROSINE REPLACEMENTS

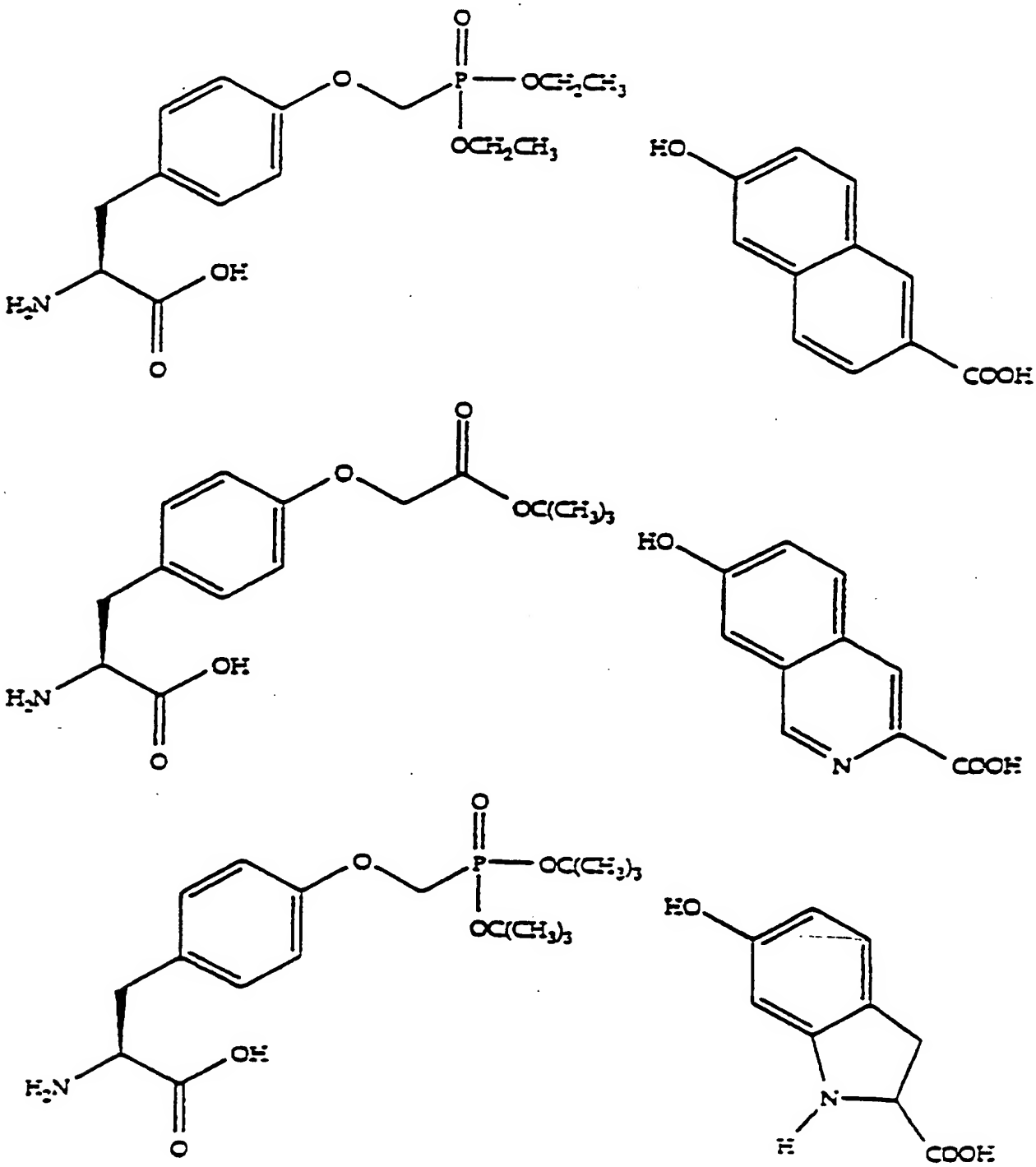


FIGURE 10(b)

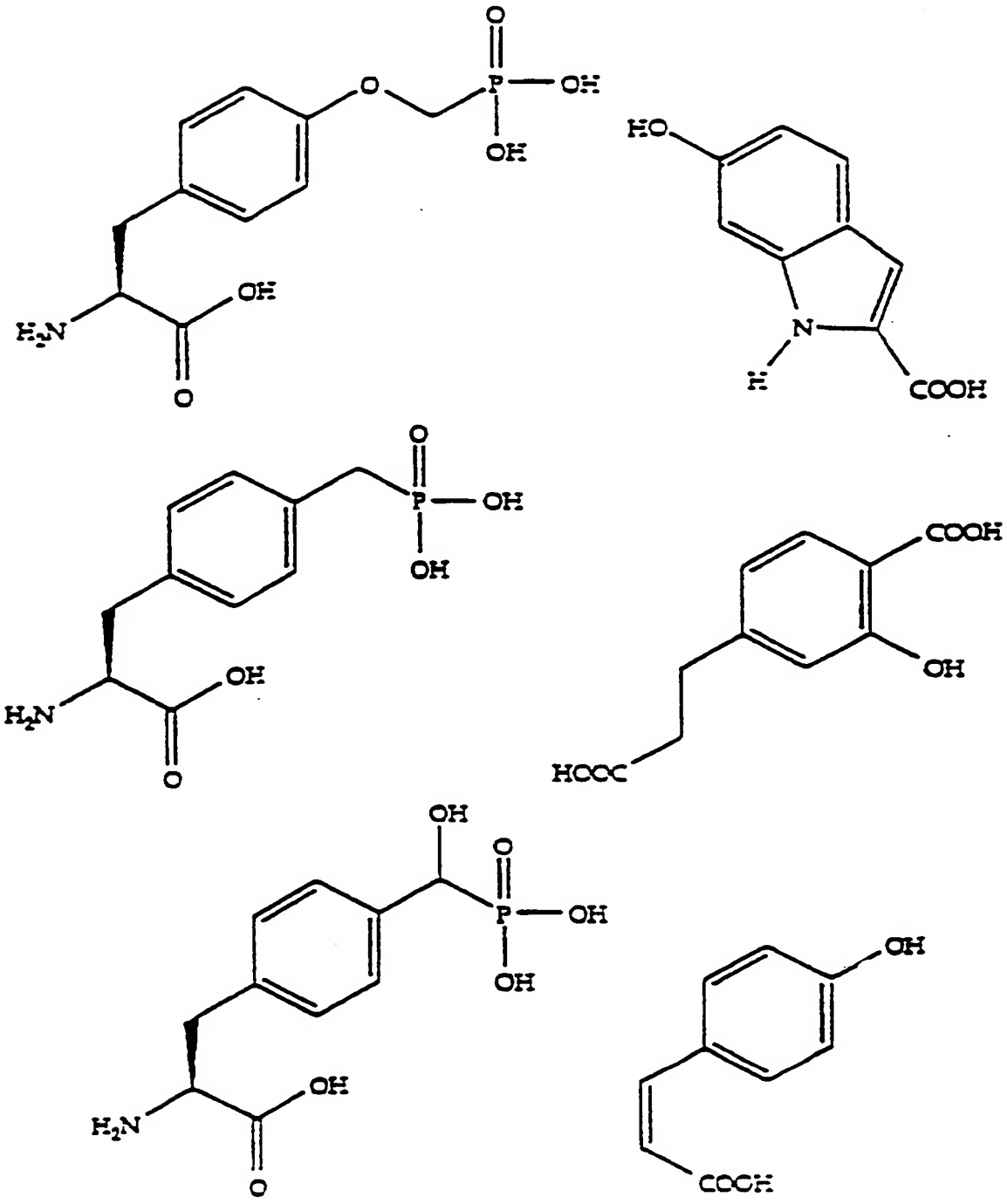


FIGURE 10(c)

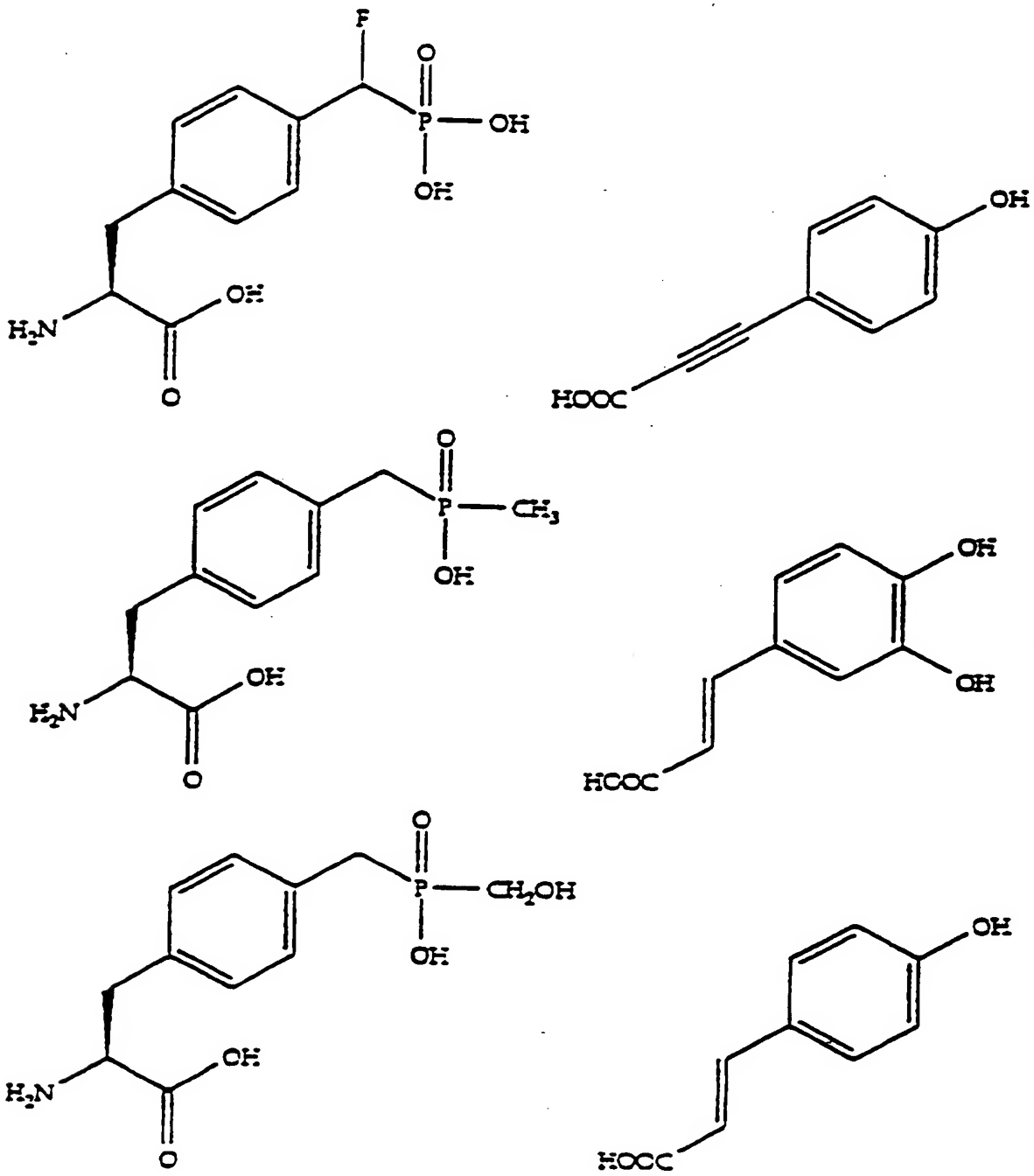


FIGURE 10(d)

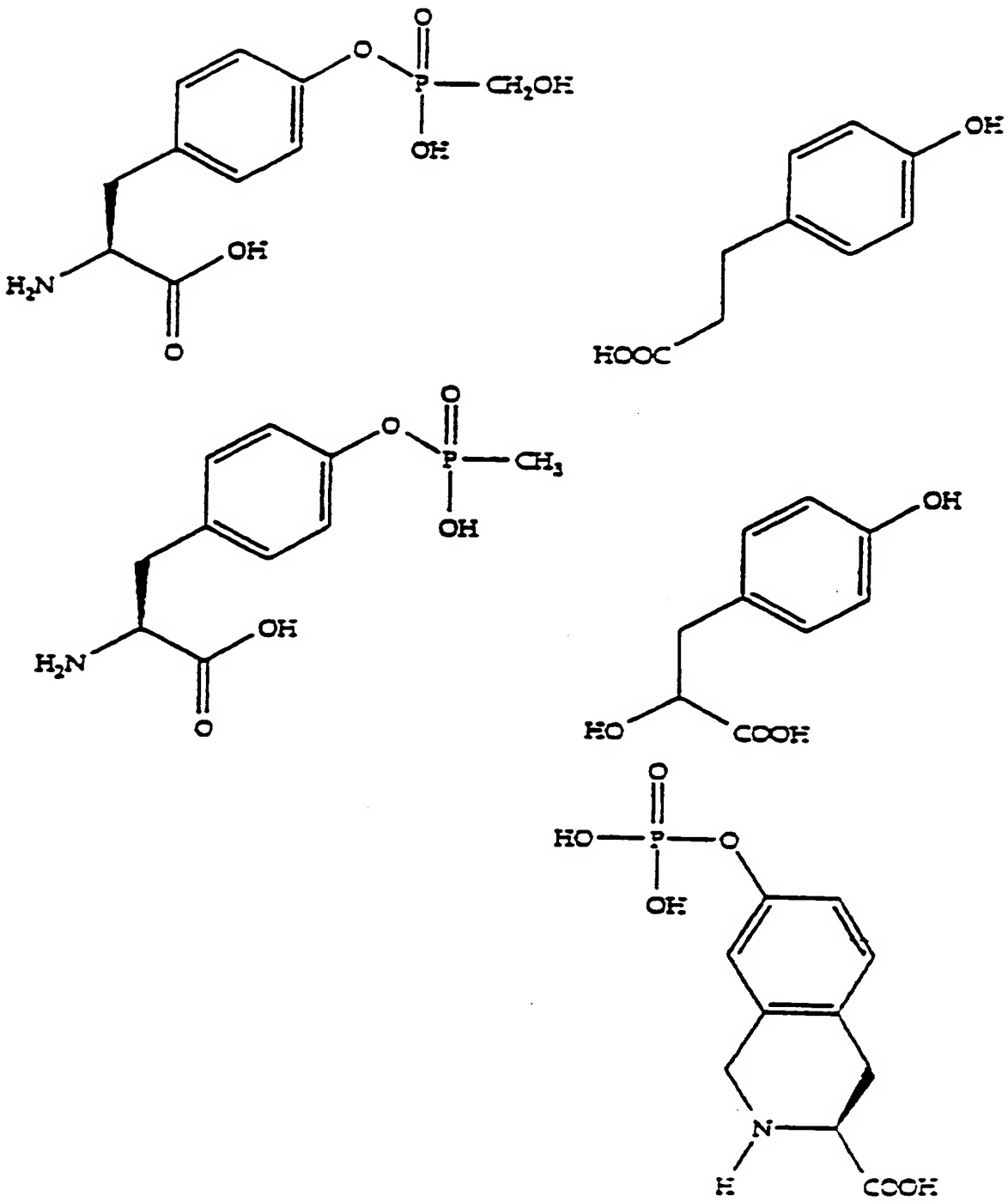


FIGURE 10(e)

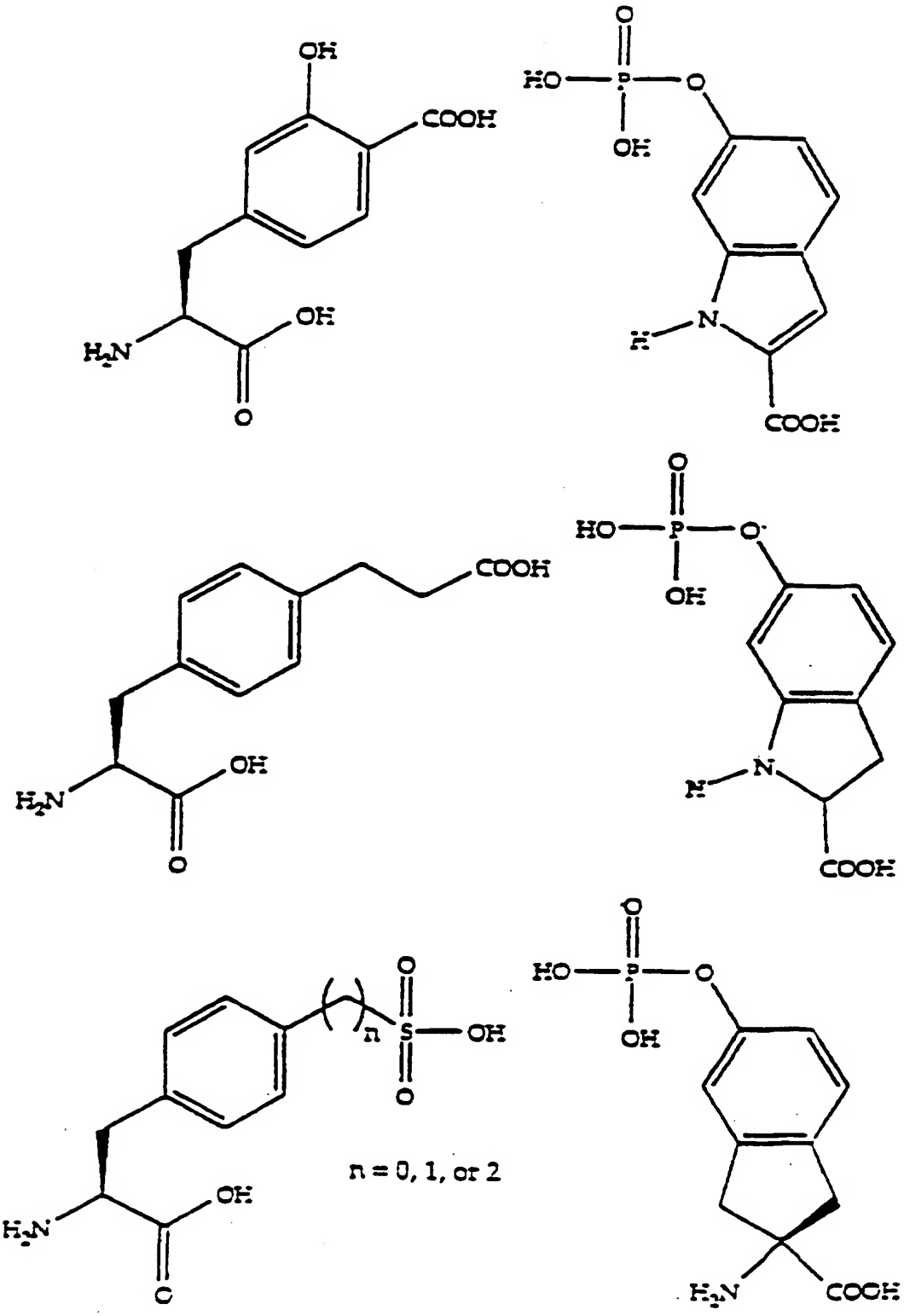
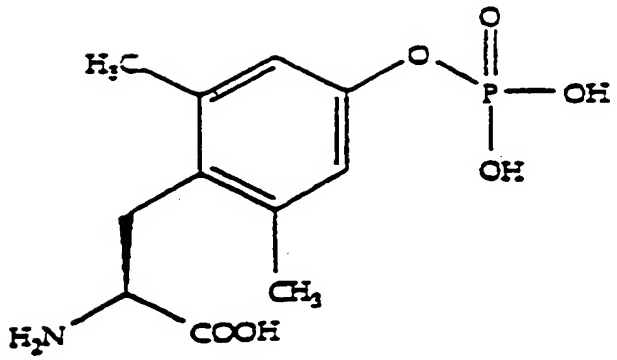
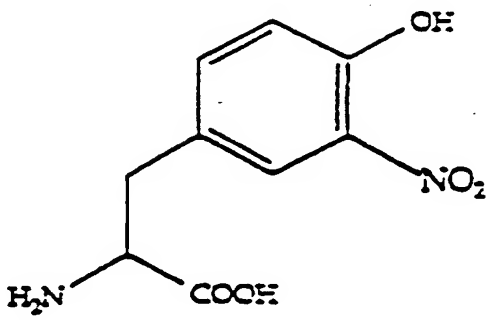
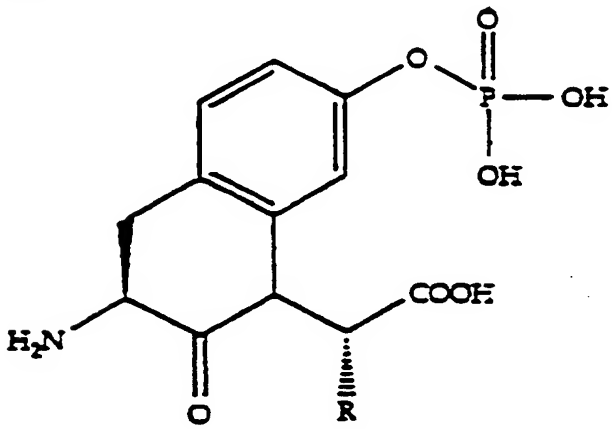
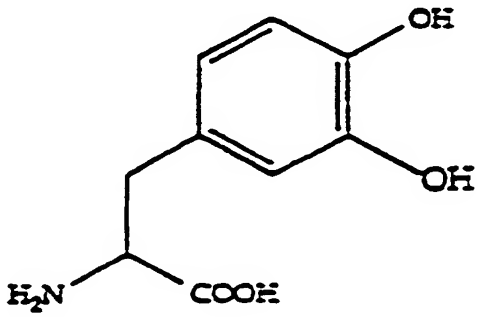


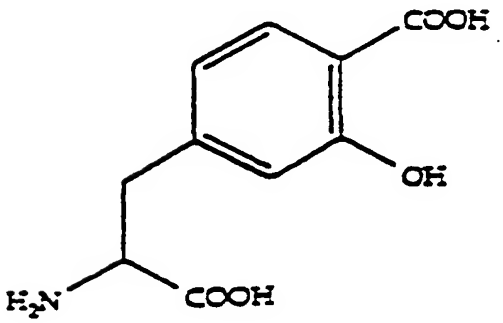
FIGURE 10(f)











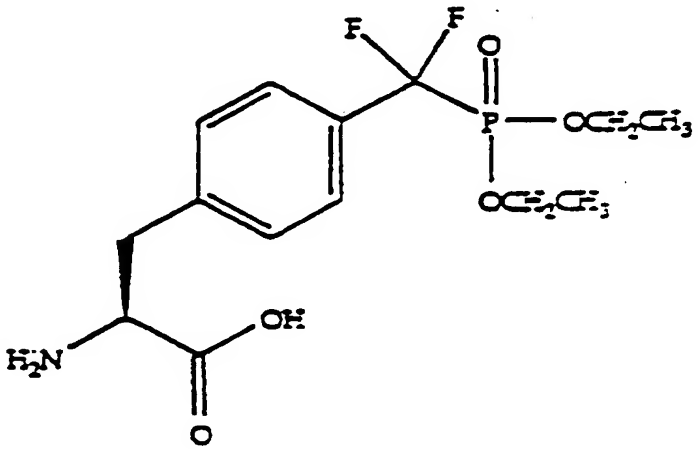


FIGURE 10(g)

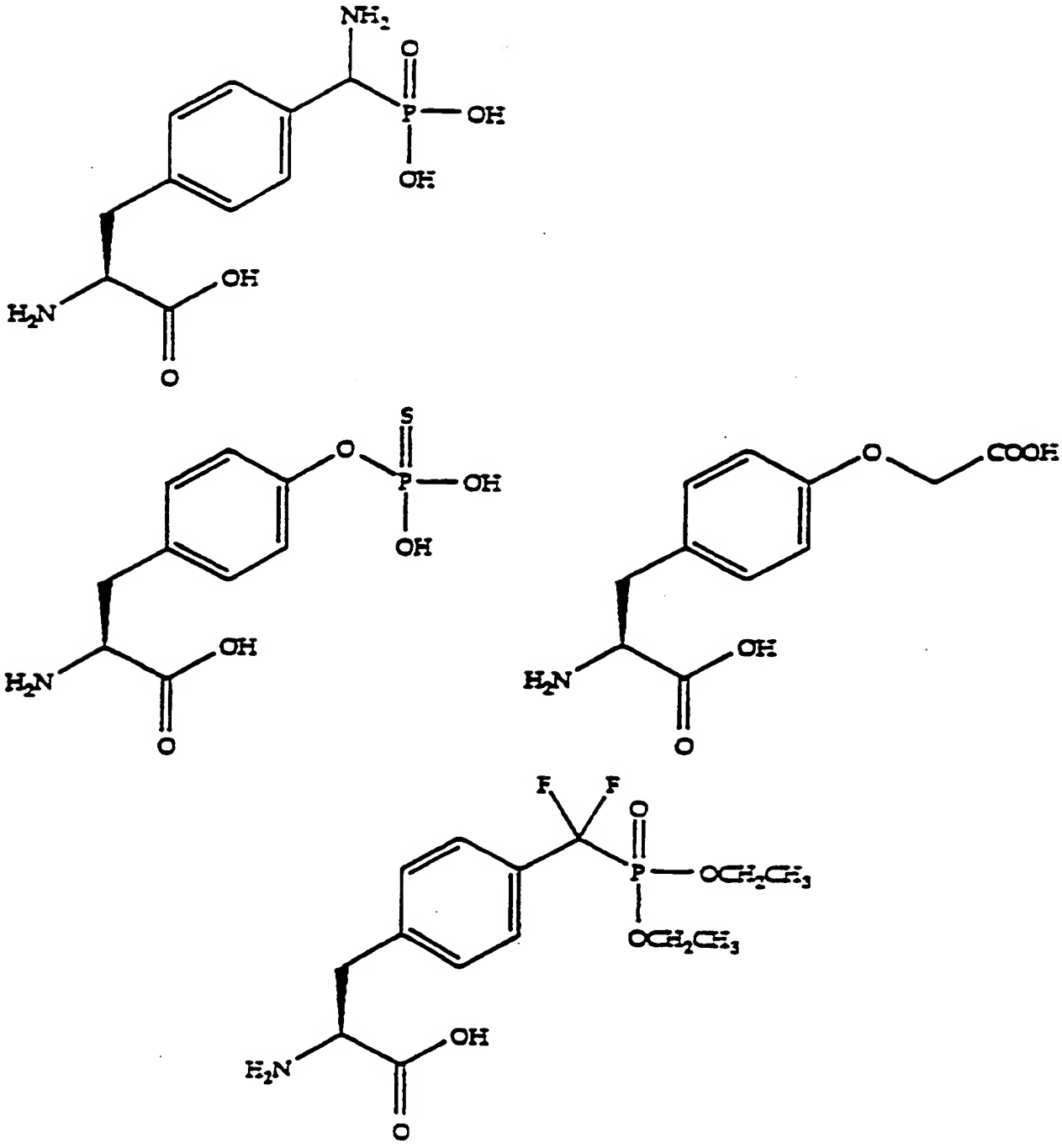

NH2
O
P
OH
OH
OH
H2N
O
S
O
P
OH
OH
OH
H2N
O
O
COOH
OH
H2N
O
F
F
O
P
OCH2CH3
OCH2CH3
OH
H2N
O

FIGURE 11(a)

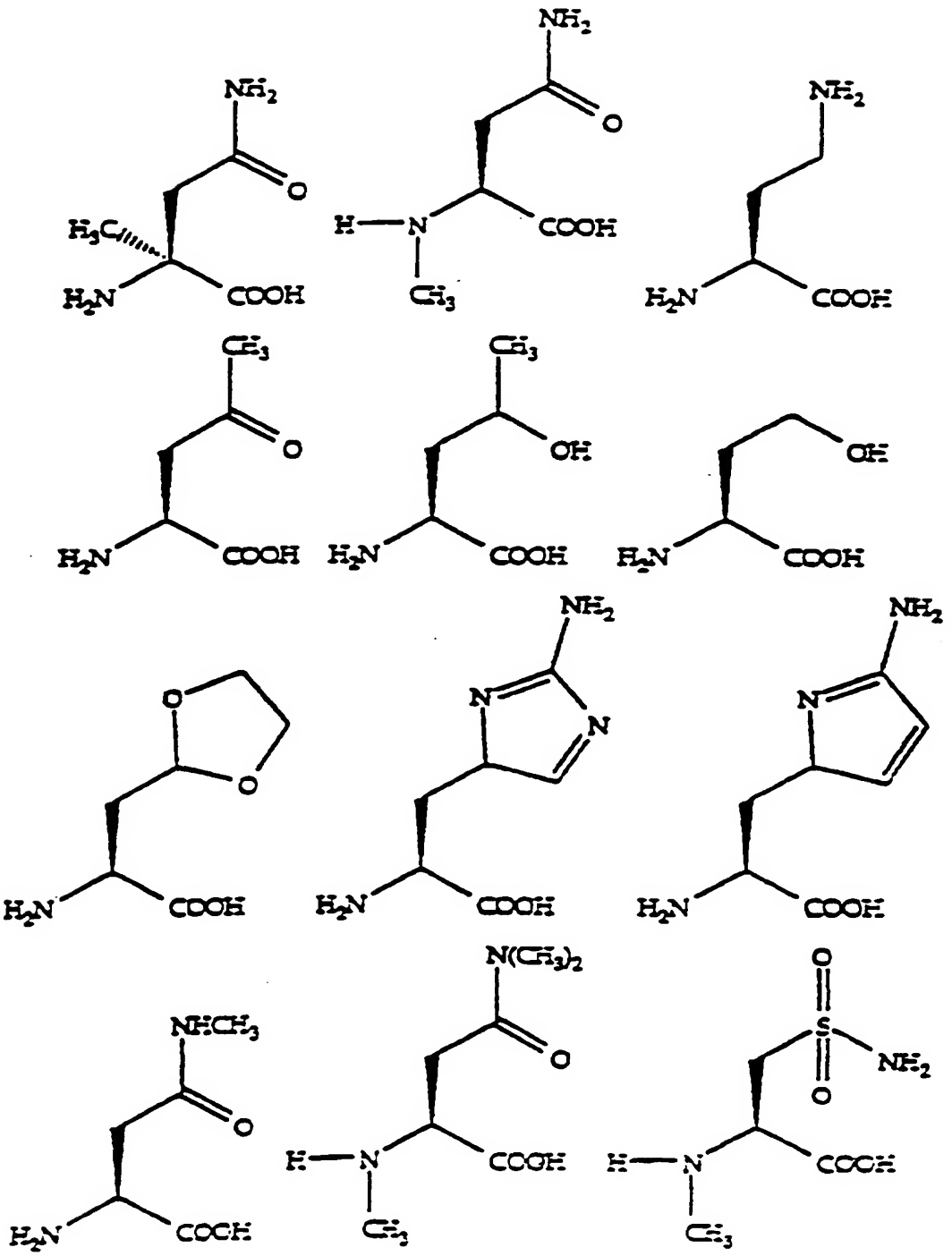


FIGURE 11(b)

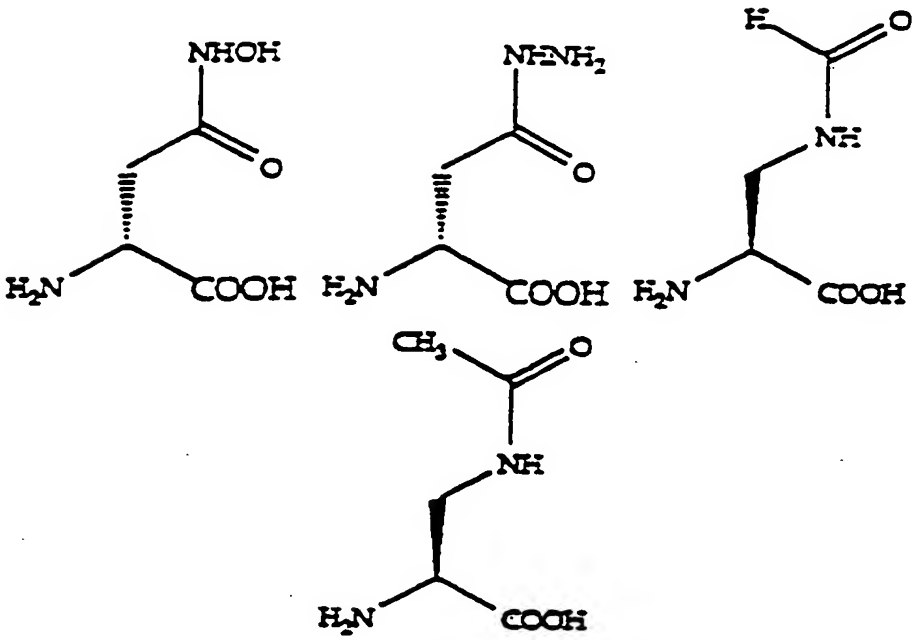


FIGURE 12

Representative Amino Acids for ESL Constructions

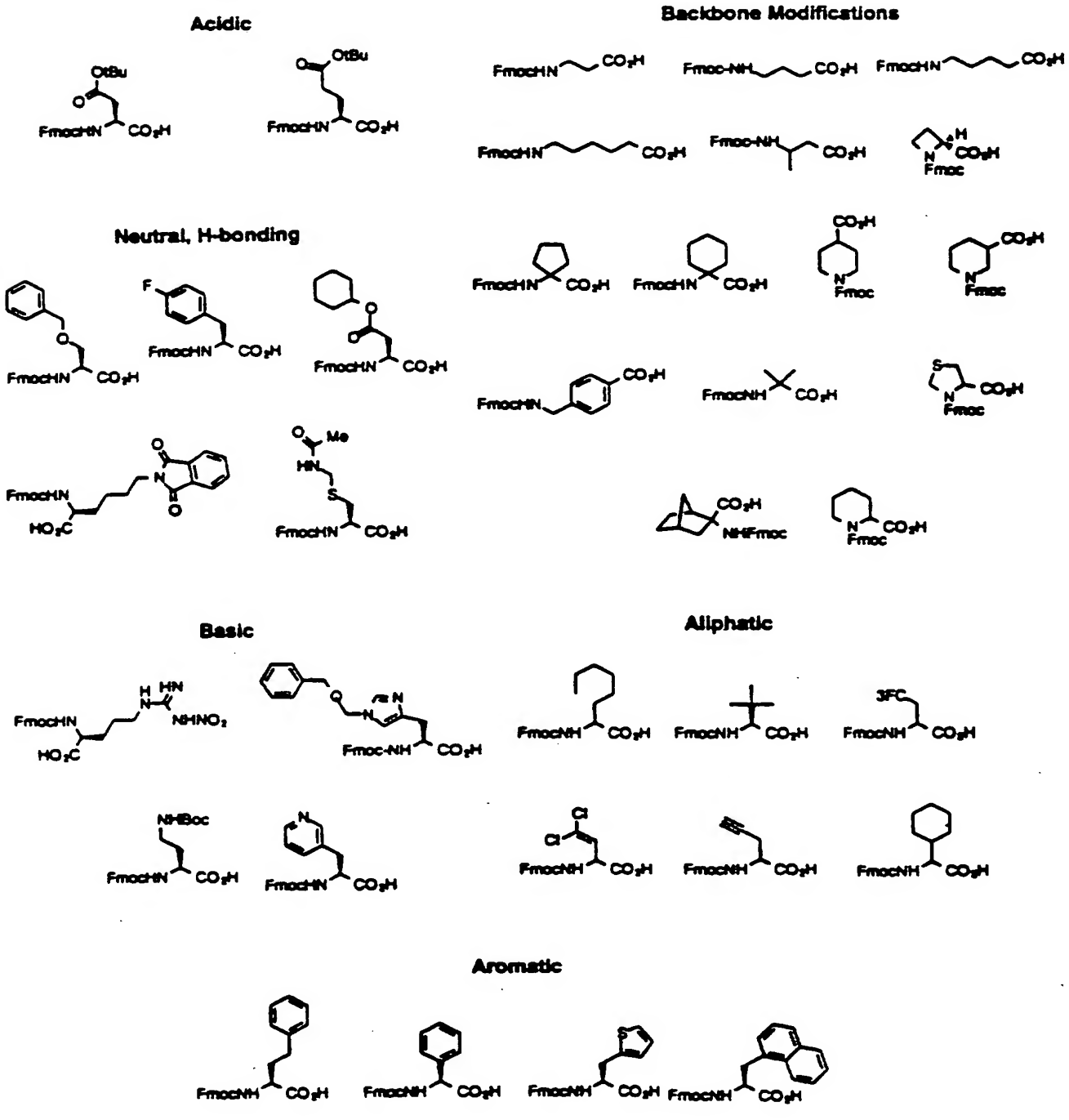


FIGURE 13(a)

Library Aromatic:

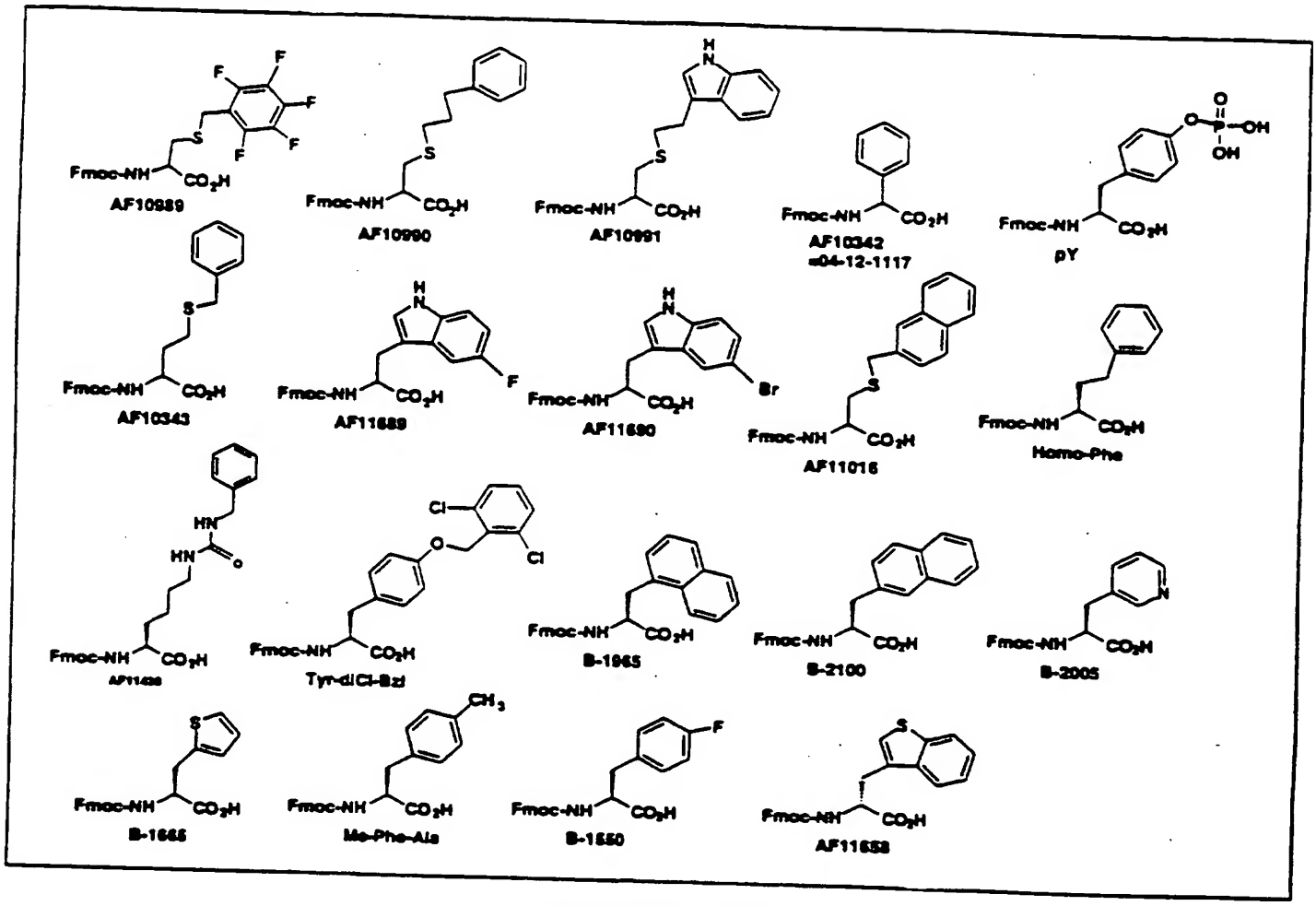


FIGURE 13(b)

Basic Building Blocks (8)

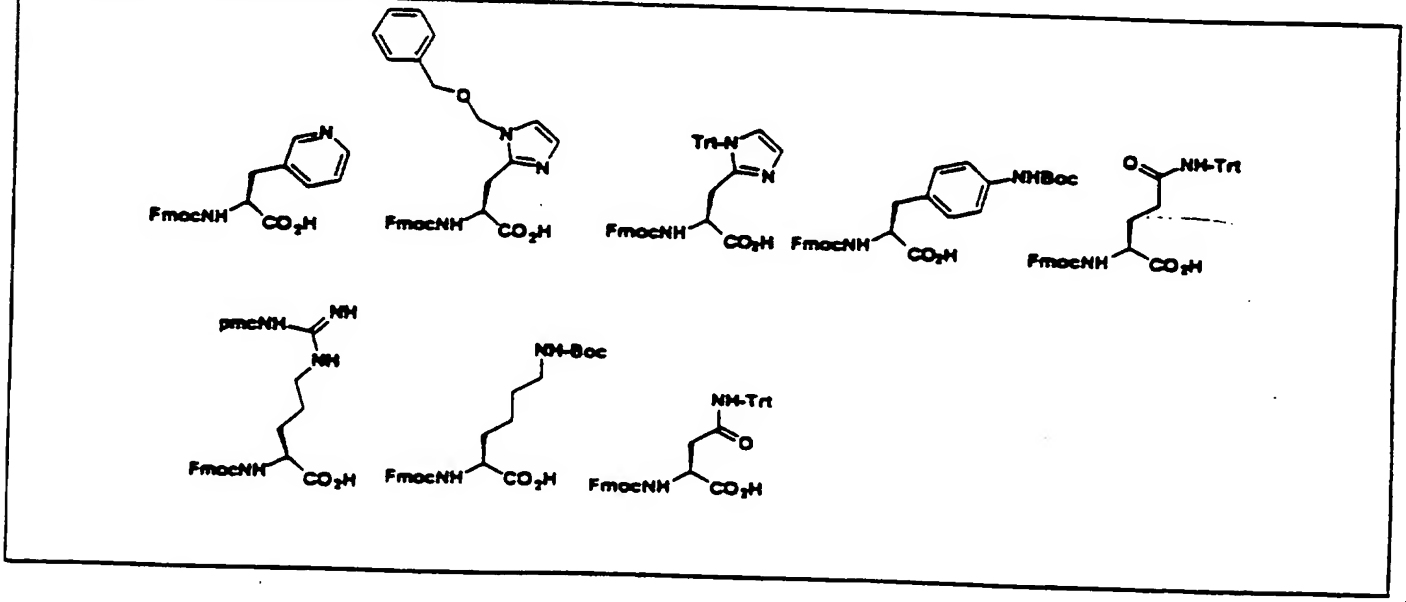


FIGURE 13(c)

Acidic Building Blocks (7)

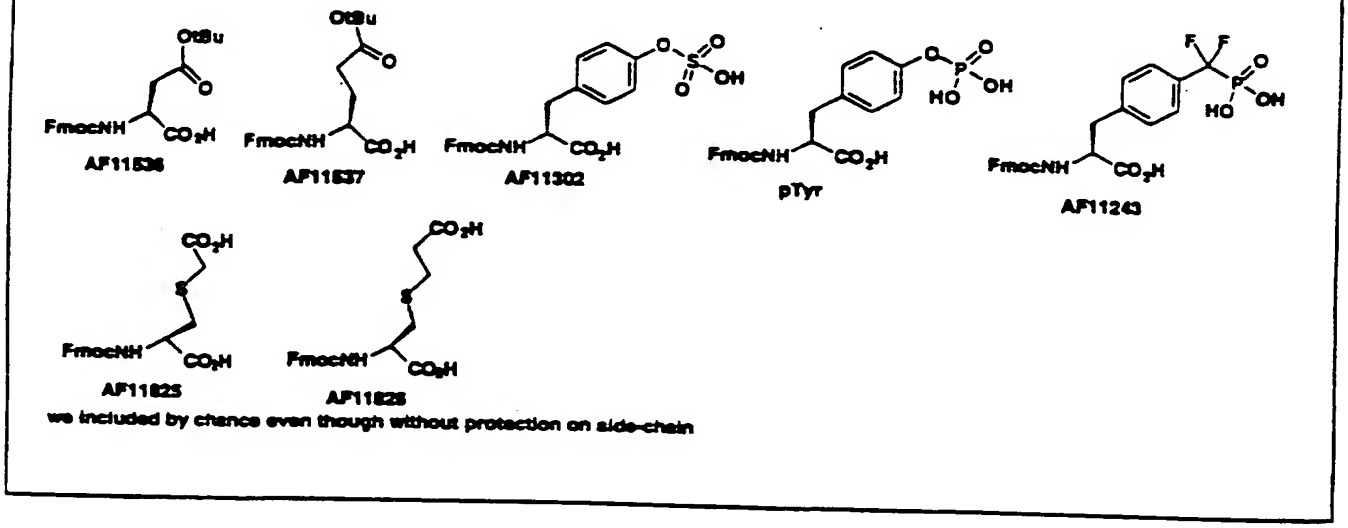
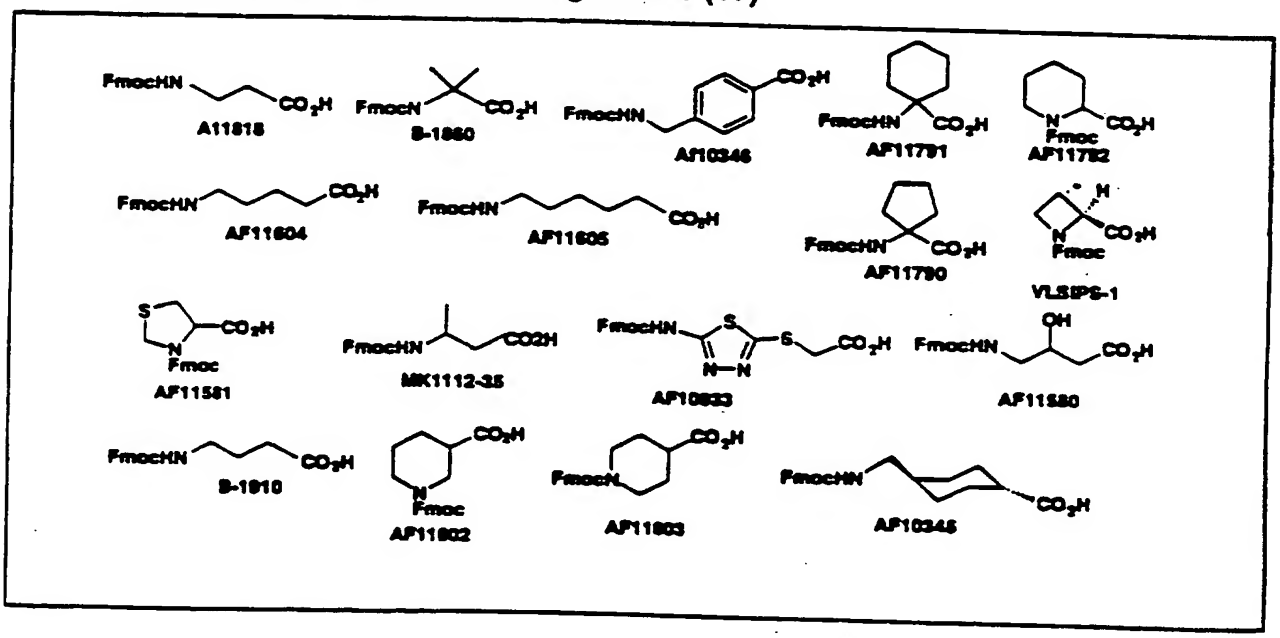


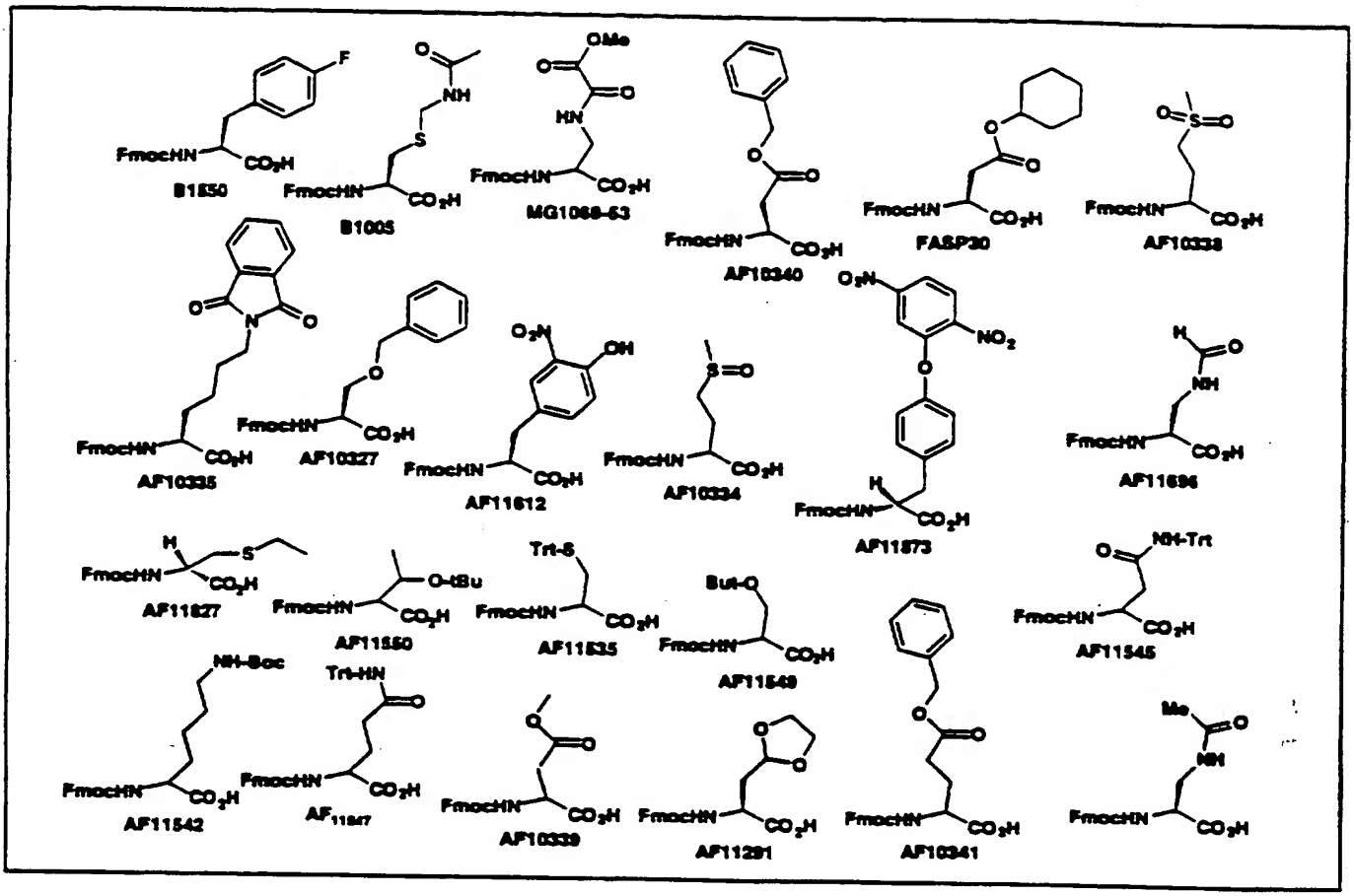
FIGURE 13(d)

Backbone Modification Building Blocks (17)



Neutral H-bonding Building Blocks (23)

FIGURE 13(e)



d-Amino acid building blocks (19)

1. Fmoc-d-ala-OH
2. Fmoc-d-gly-OH
3. Fmoc-d-ile-OH
4. Fmoc-d-leu-OH
5. Fmoc-d-phe-OH
6. Fmoc-d-val-OH
7. Fmoc-d-pro-OH
8. Fmoc-d-met-OH
9. Fmoc-d-thr(tBu)-OH
10. Fmoc-d-ser(tBu)-OH
11. Fmoc-d-trp(Boc)-OH
12. Fmoc-d-arg(N^{g}Mtr)-OH
13. Fmoc-d-gln(trt)-OH
14. Fmoc-d-lys(Boc)-OH
15. Fmoc-d-his(trt)-OH
16. Fmoc-d-asp(beta tBU)-OH
17. Fmoc-d-glu(gamma tBu)-OH
18. Fmoc-d-asn(trt)-OH
19. Fmoc-d-tyr(tBu)-OH

FIGURE 13(f)

L-Amino acid building blocks (19)

1. Fmoc-Ala-OH
2. Fmoc-Gly-OH
3. Fmoc-Ile-OH
4. Fmoc-Leu-OH
5. Fmoc-Phe-OH
6. Fmoc-Val-OH
7. Fmoc-Pro-OH
8. Fmoc-Met-OH
9. Fmoc-Thr(tBu)-OH
10. Fmoc-Ser(tBu)-OH
11. Fmoc-Trp(Boc)-OH
12. Fmoc-Arg(N^{g}pmc)-OH
13. Fmoc-Gln(trt)-OH
14. Fmoc-Lys(Boc)-OH
15. Fmoc-His(trt)-OH
16. Fmoc-Asp(beta tBU)-OH
17. Fmoc-Glu(gamma tBu)-OH
18. Fmoc-Asn(trt)-OH
19. Fmoc-Tyr(tBu)-OH

FIGURE 13(g)

Hydrophobic and Aliphatic building blocks (17)

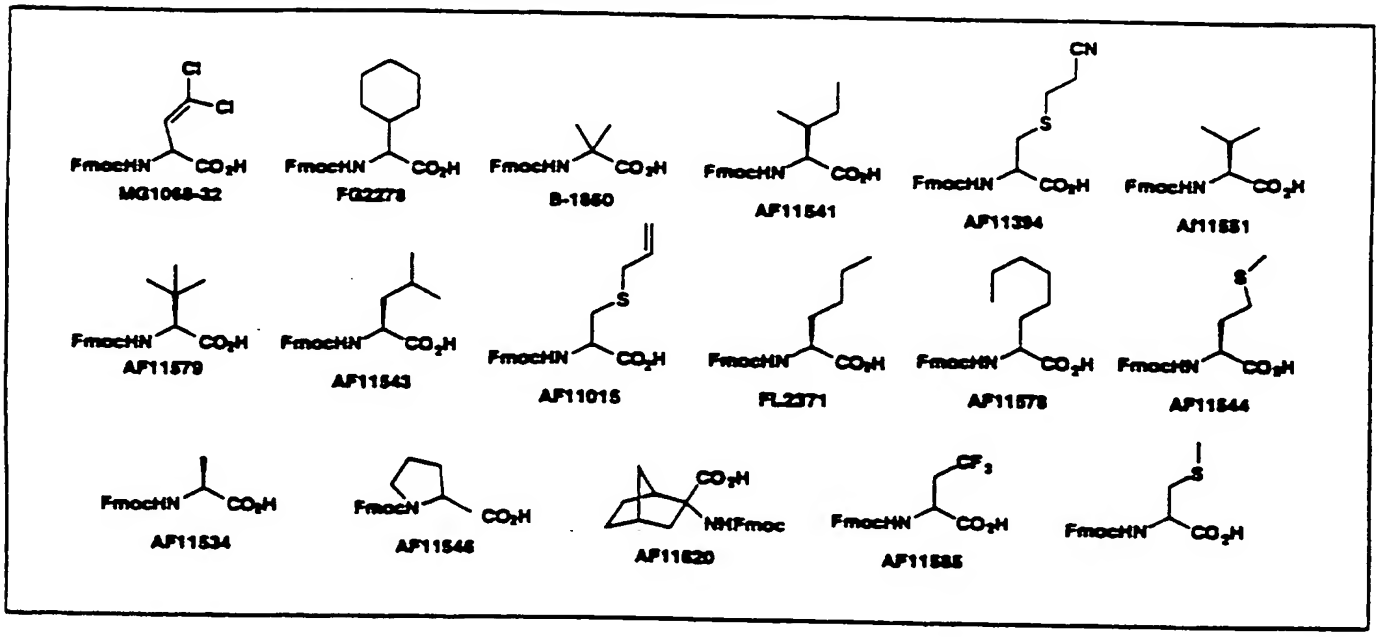


FIGURE 13(h)

Lysine der. Library (19)

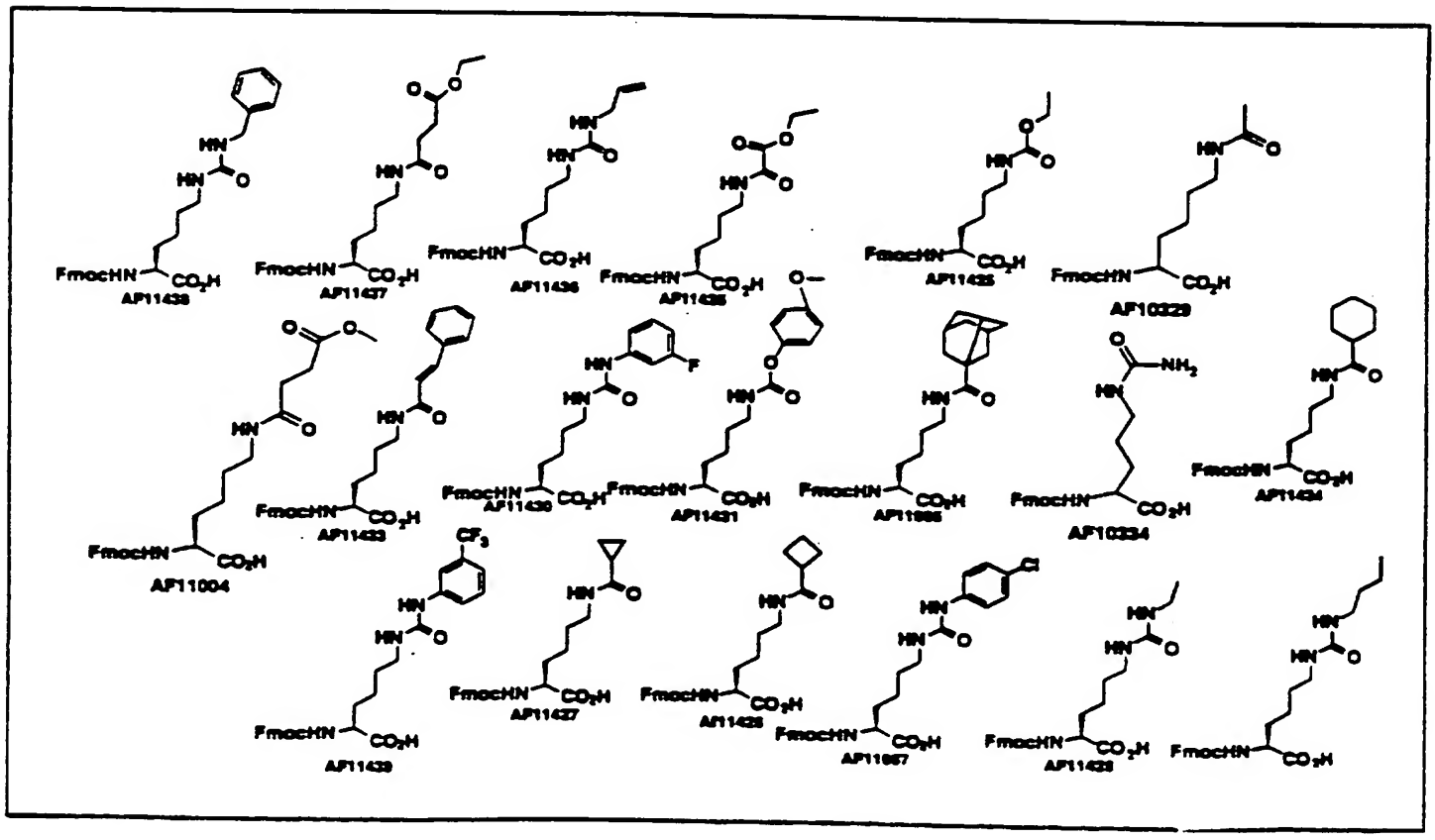
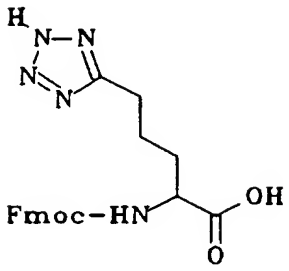


FIGURE 13(i)

FIGURE 14



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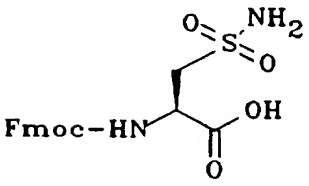

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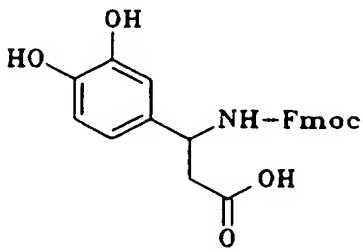

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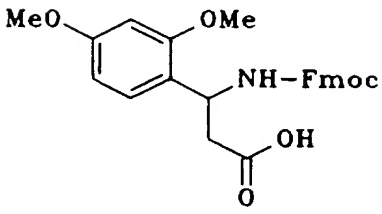

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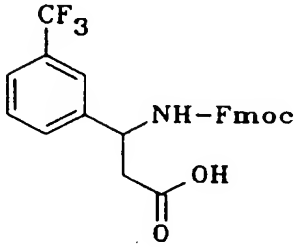

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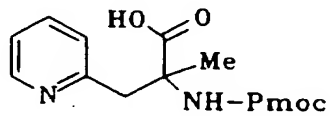

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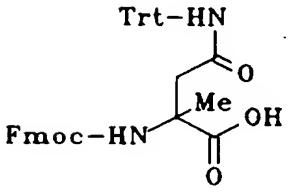

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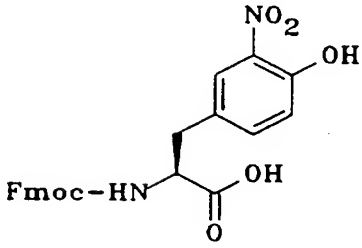

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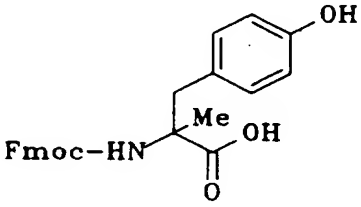

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FIGURE 15

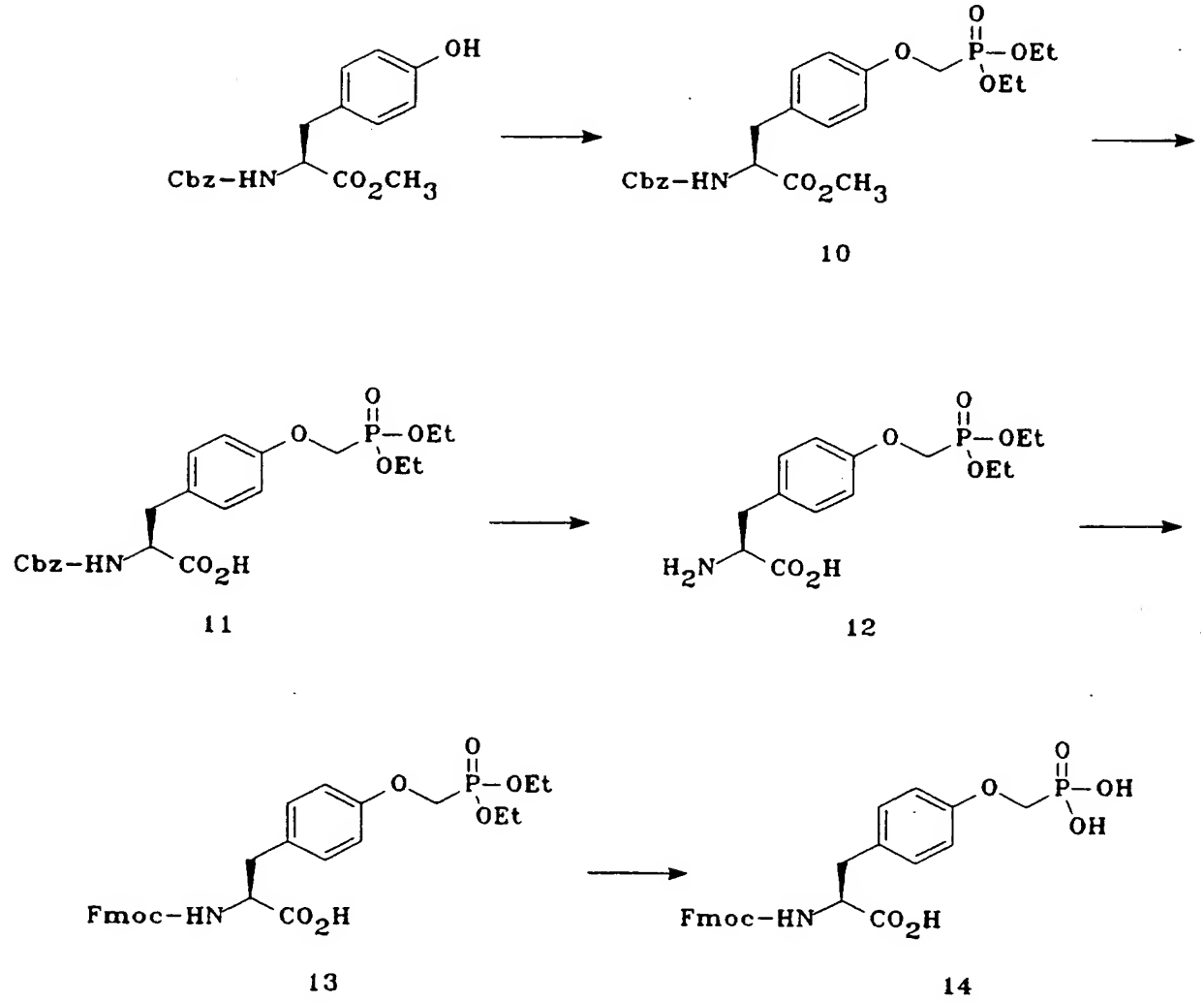


FIGURE 16

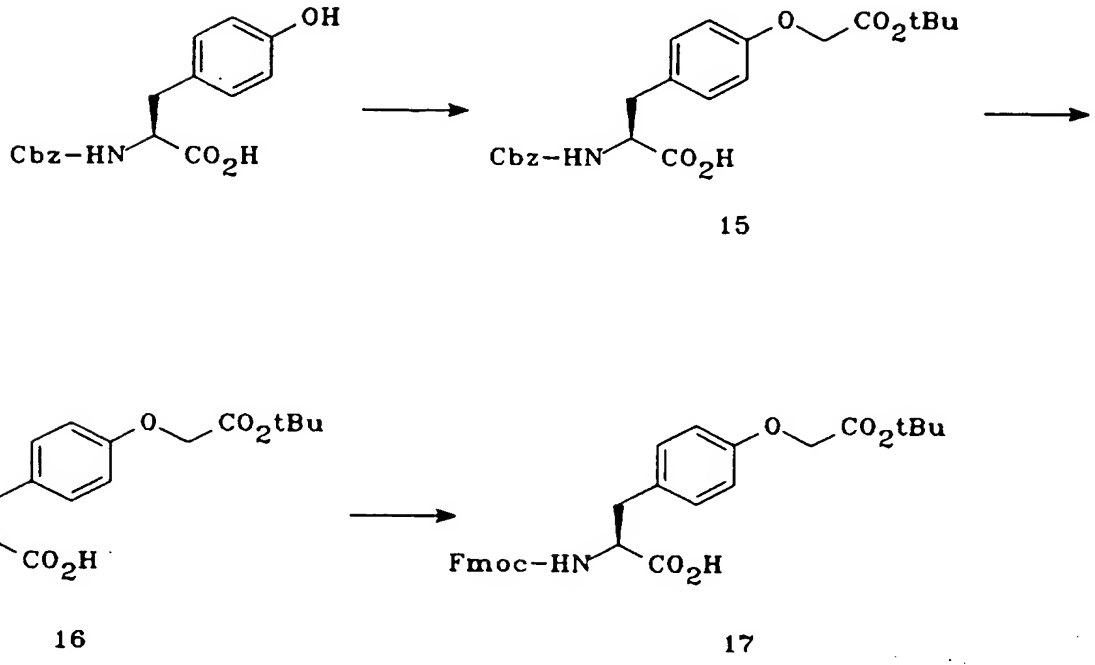

OH
Cbz-HN
CO2H
O
CO2tBu
Cbz-HN
CO2H
15
O
CO2tBu
H2N
CO2H
16
O
CO2tBu
Fmoc-HN
CO2H
17

FIGURE 17

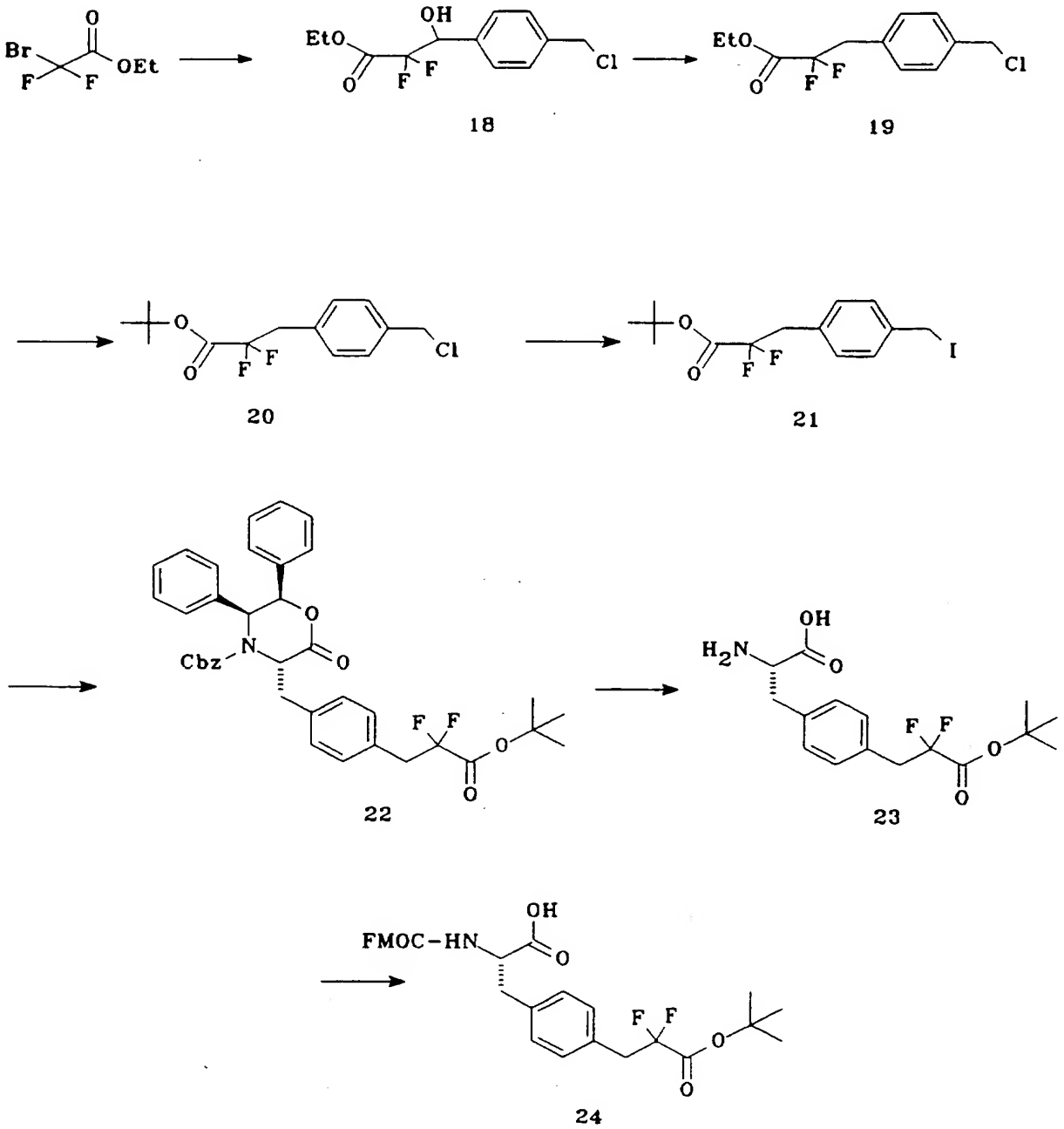


FIGURE 18

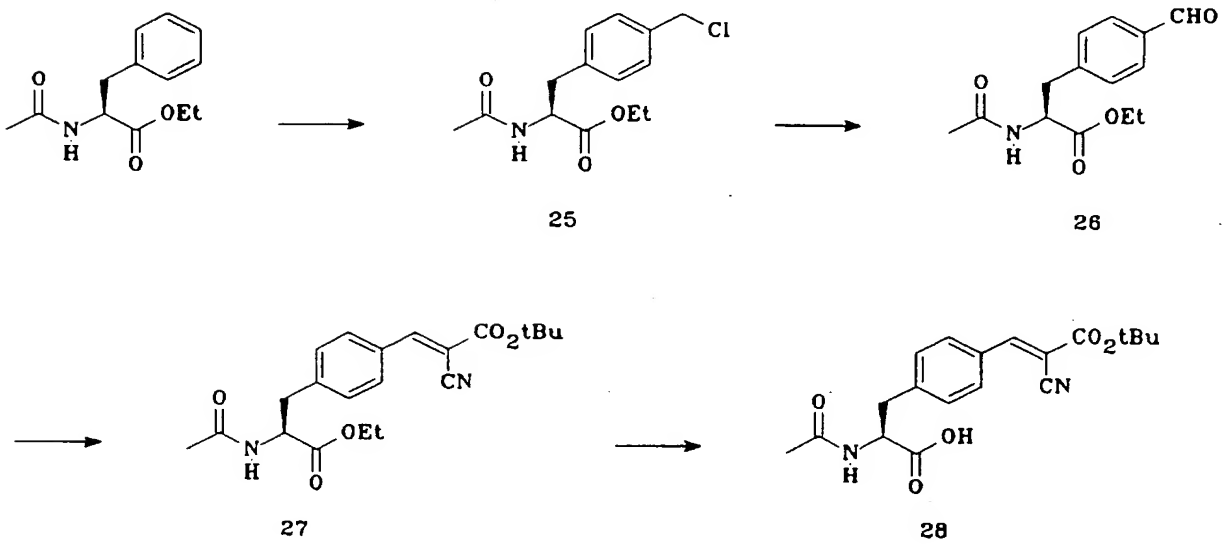


FIGURE 19

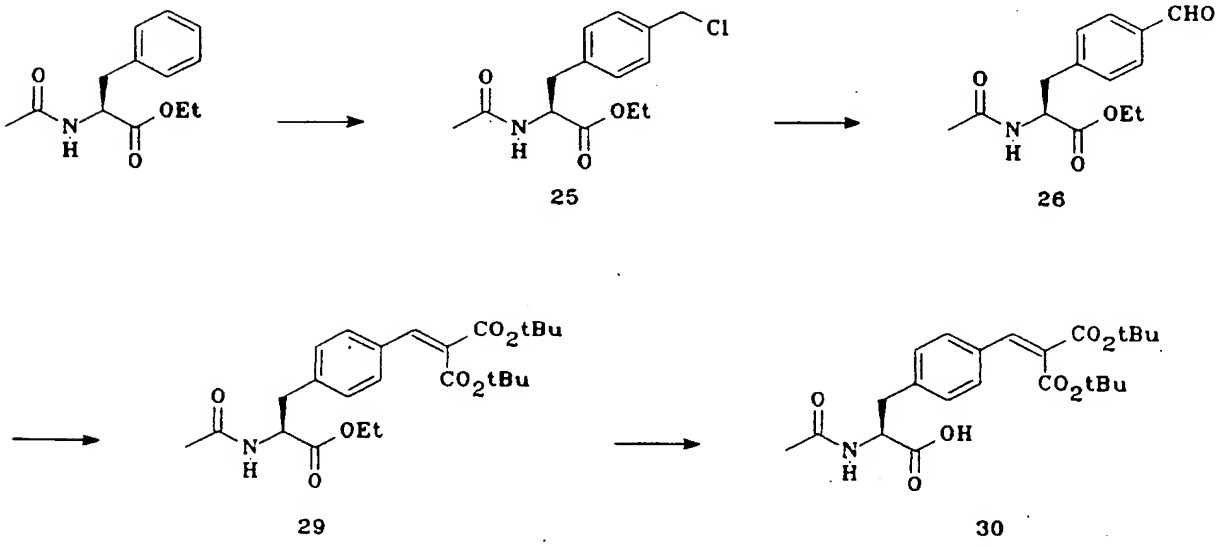


FIGURE 20

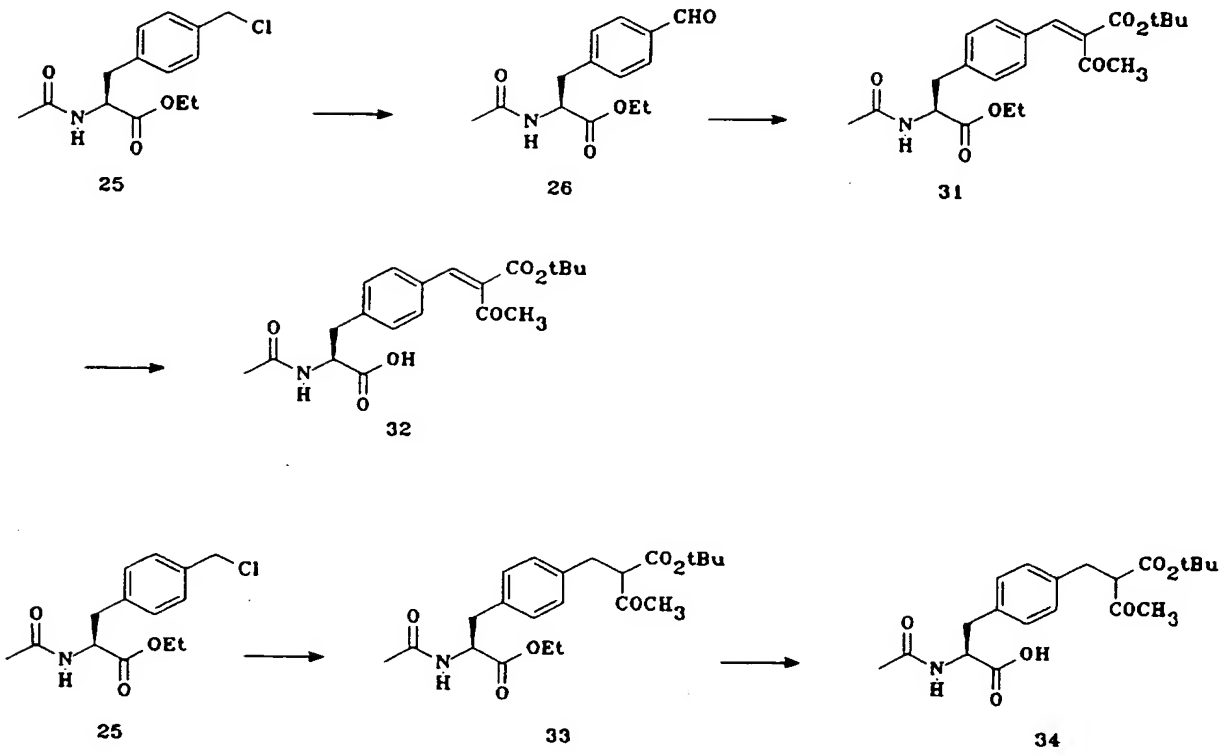


FIGURE 21

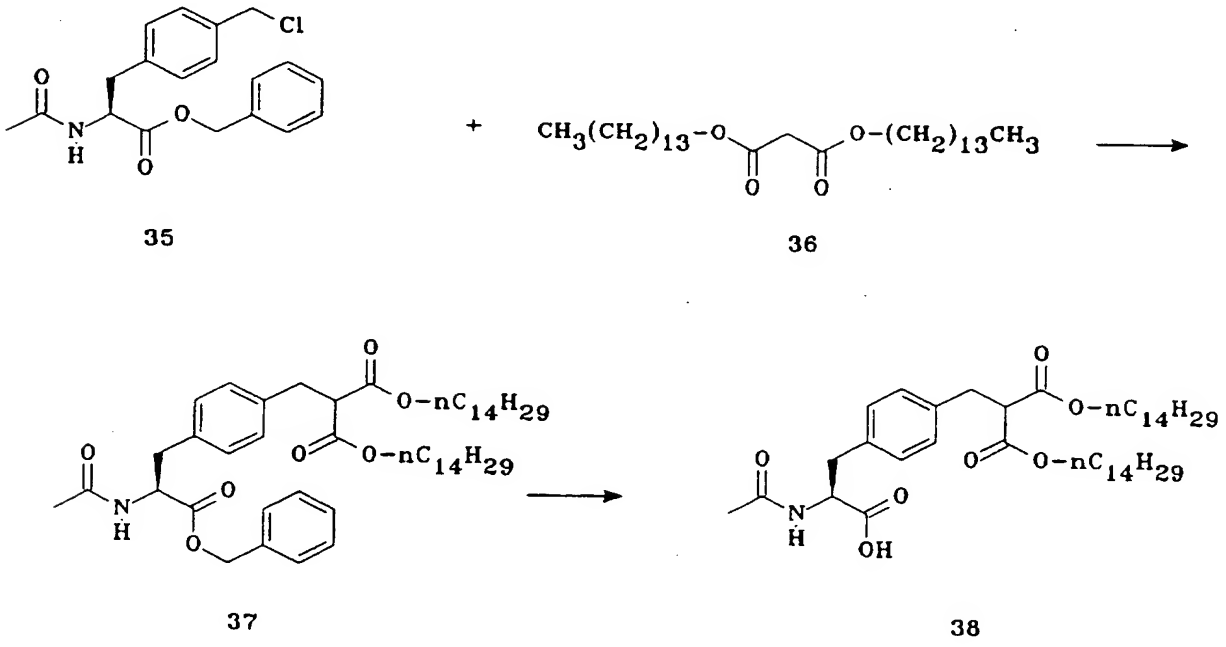


FIGURE 22

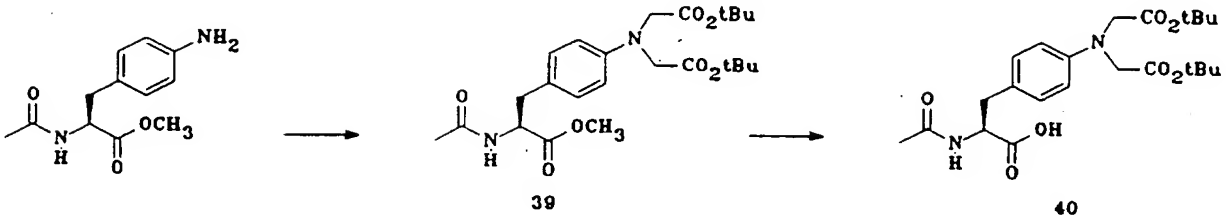


FIGURE 23

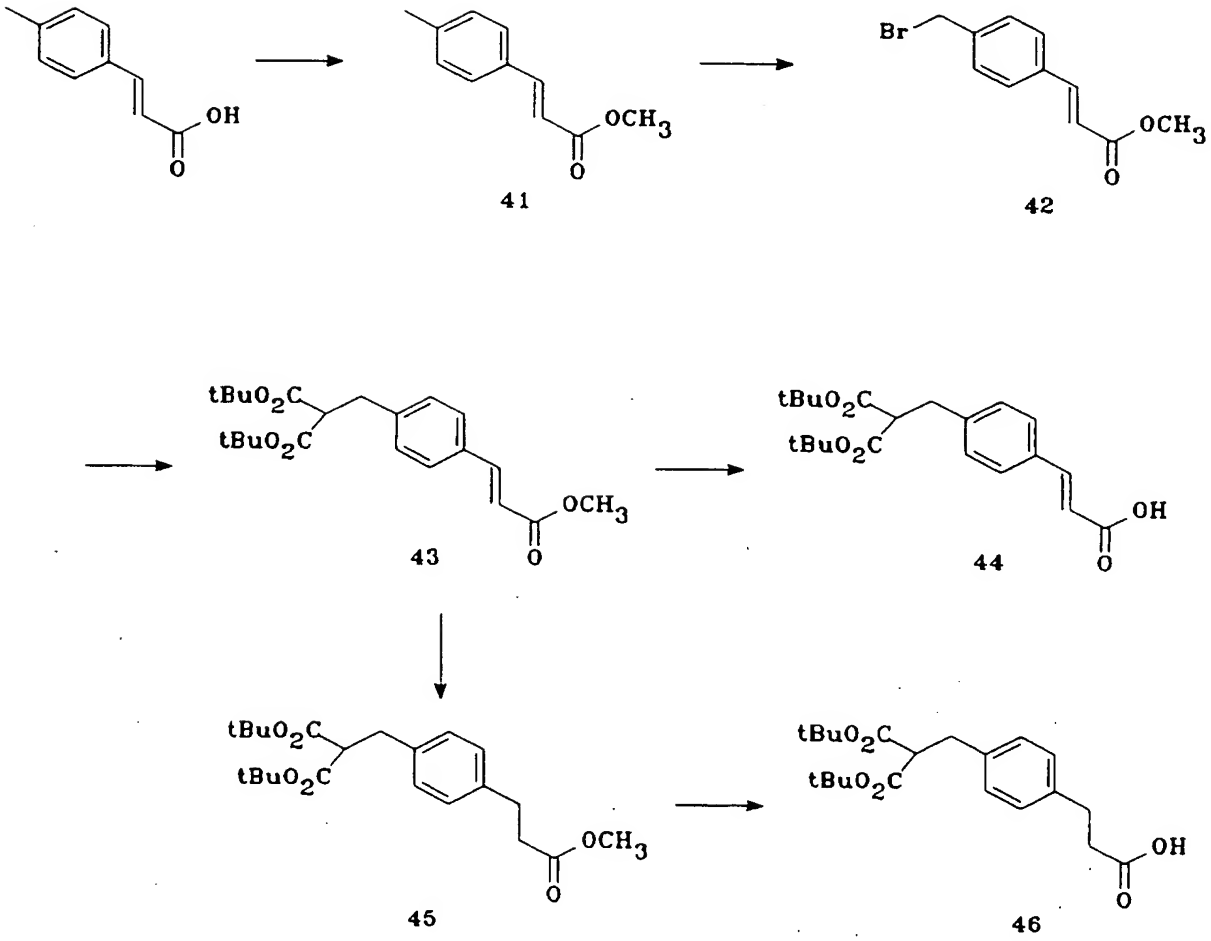


FIGURE 24

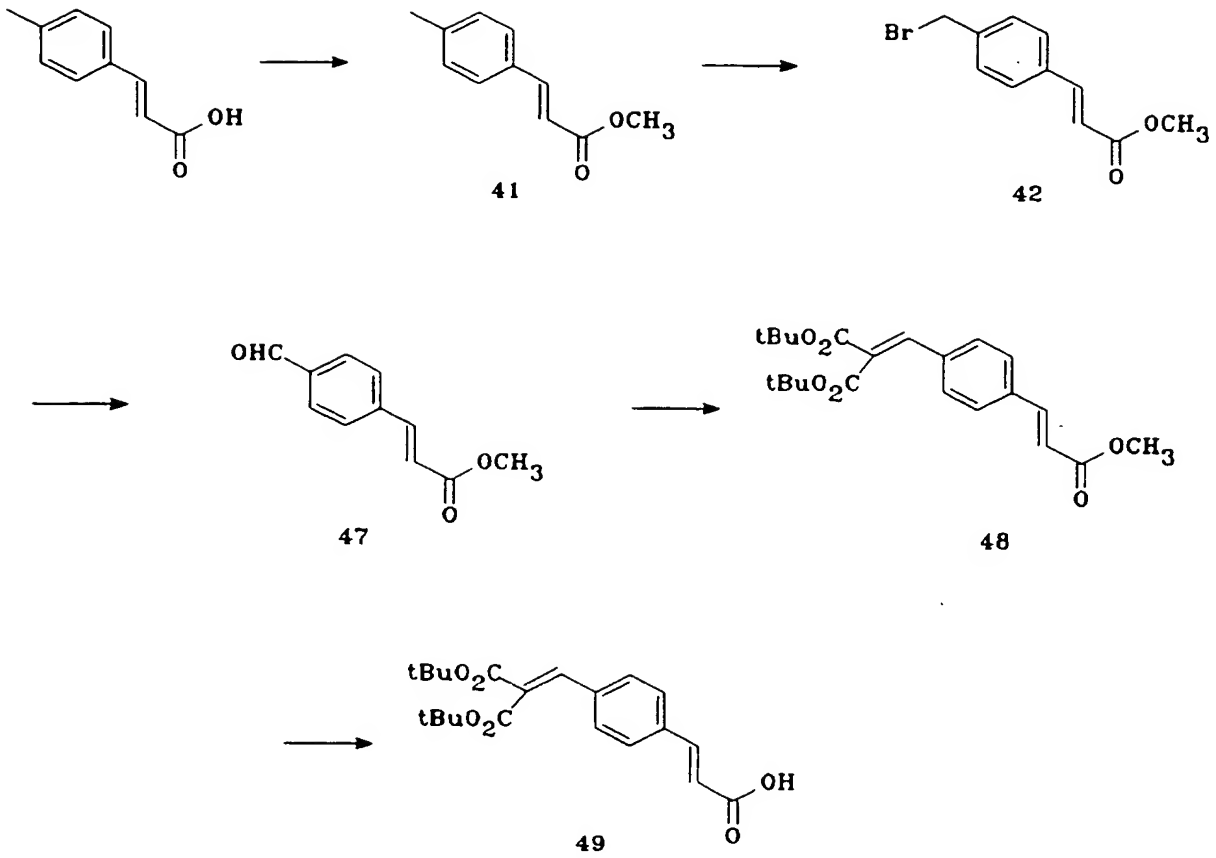


FIGURE 25

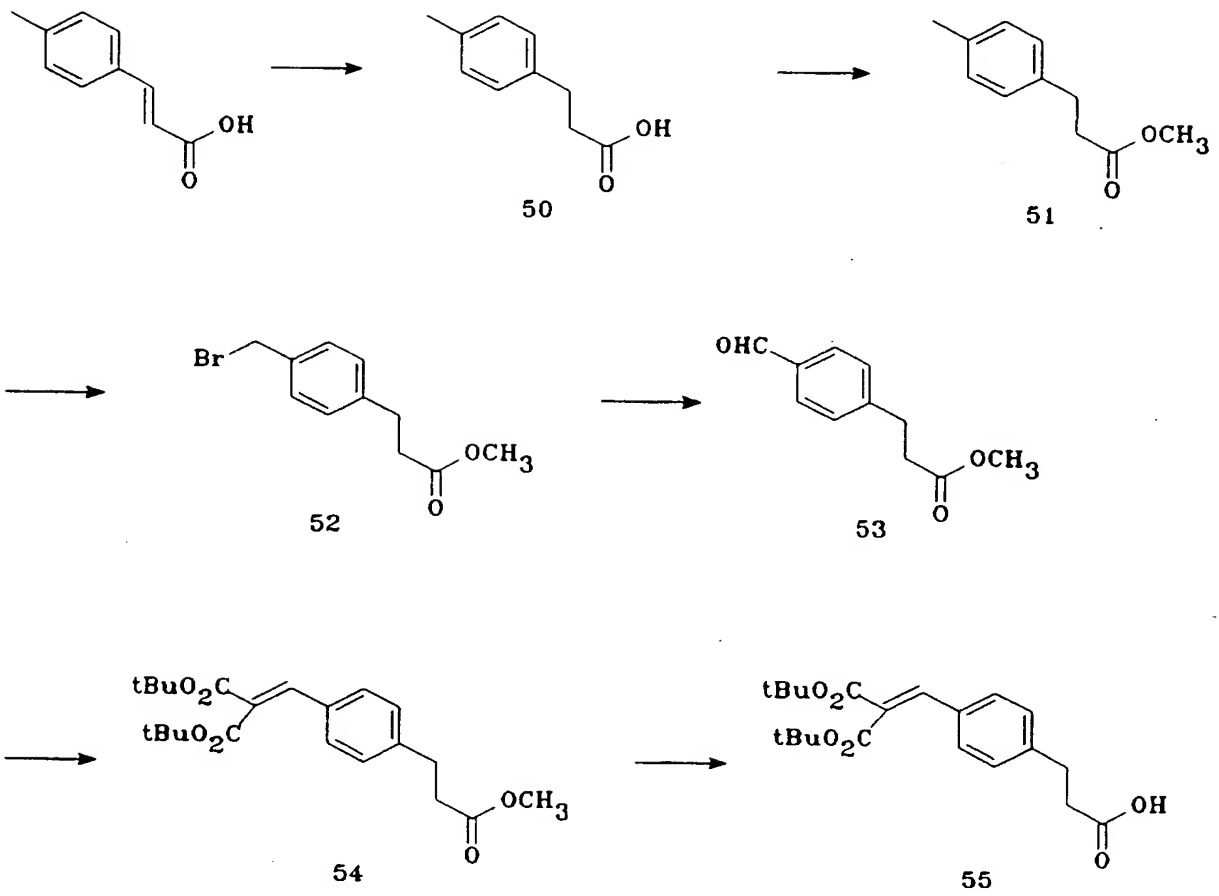


FIGURE 26

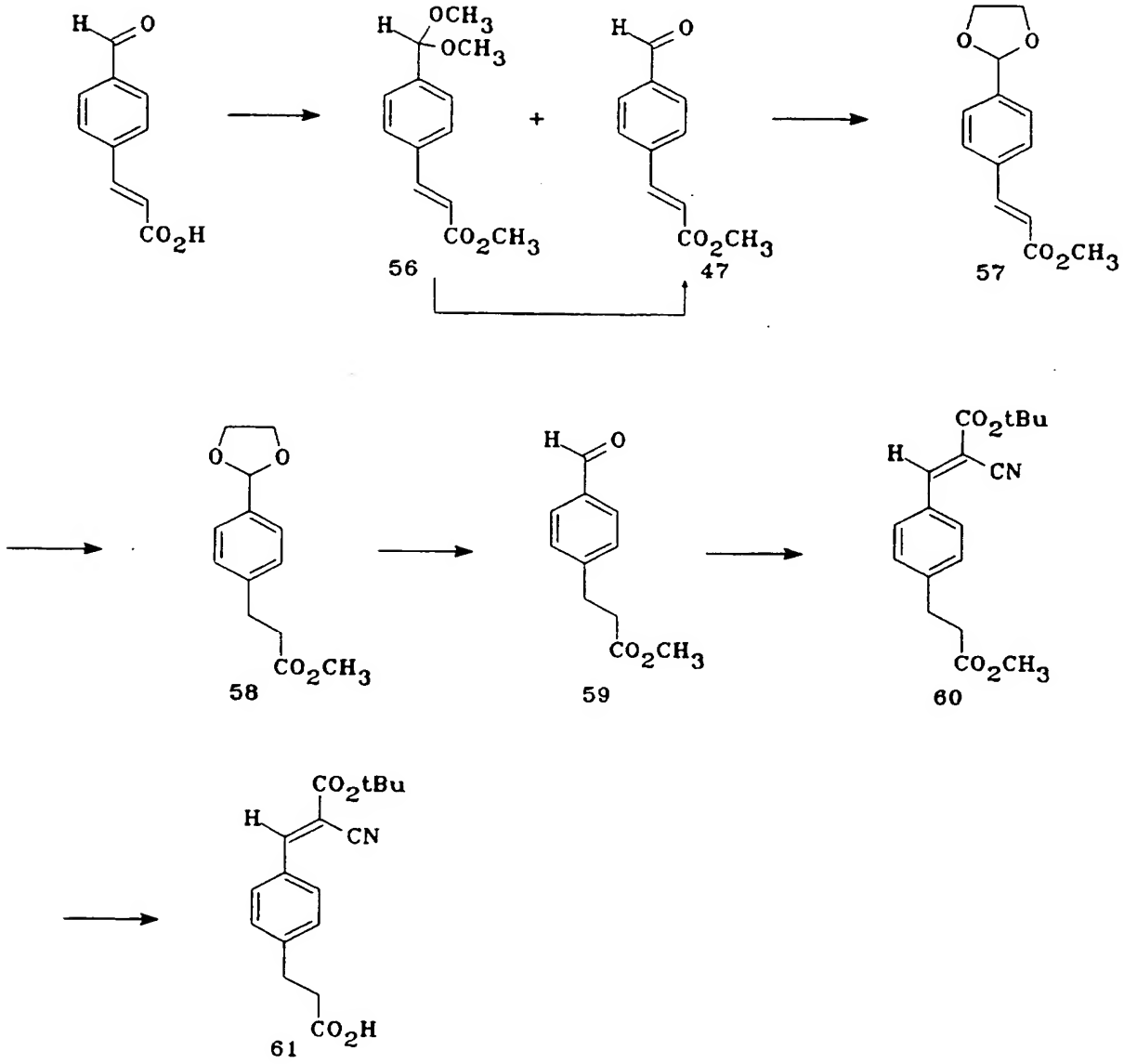


FIGURE 27

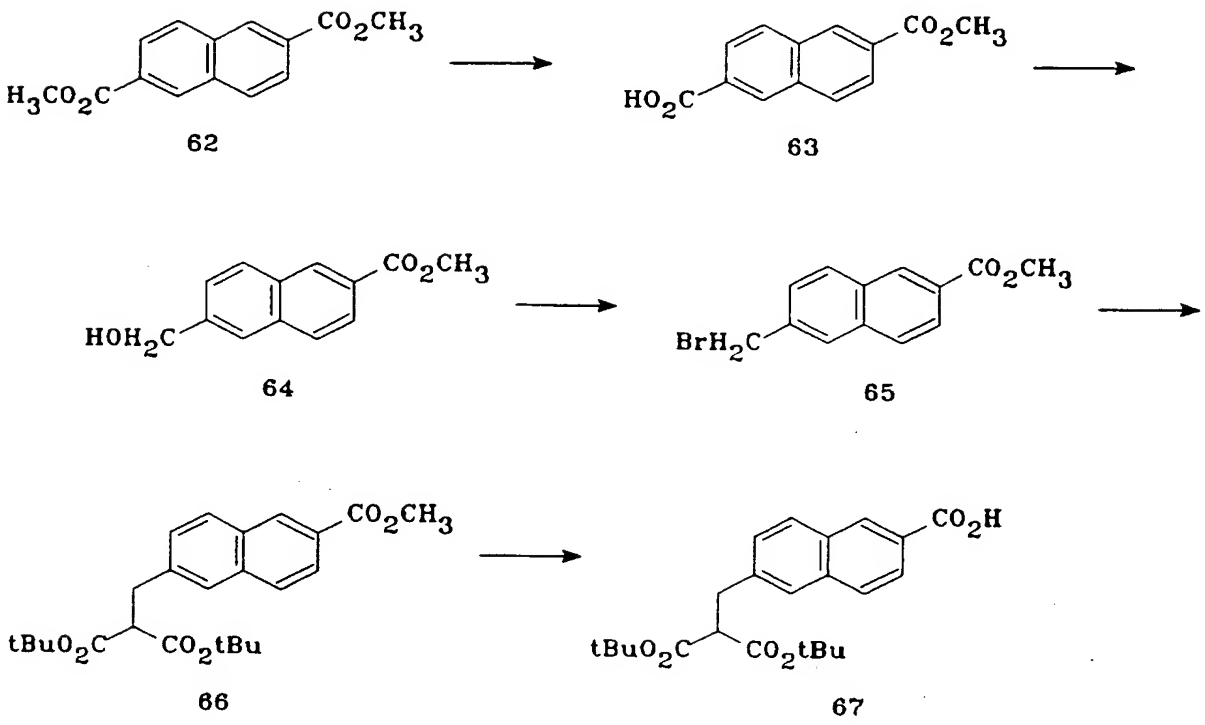

CO_2CH_3
H_3CO_2C
62
CO_2CH_3
HO_2C
63
CO_2CH_3
HOH_2C
64
CO_2CH_3
BrH_2C
65
CO_2CH_3
$tBuO_2C$
CO_2tBu
66
CO_2H
$tBuO_2C$
CO_2tBu
67

FIGURE 28

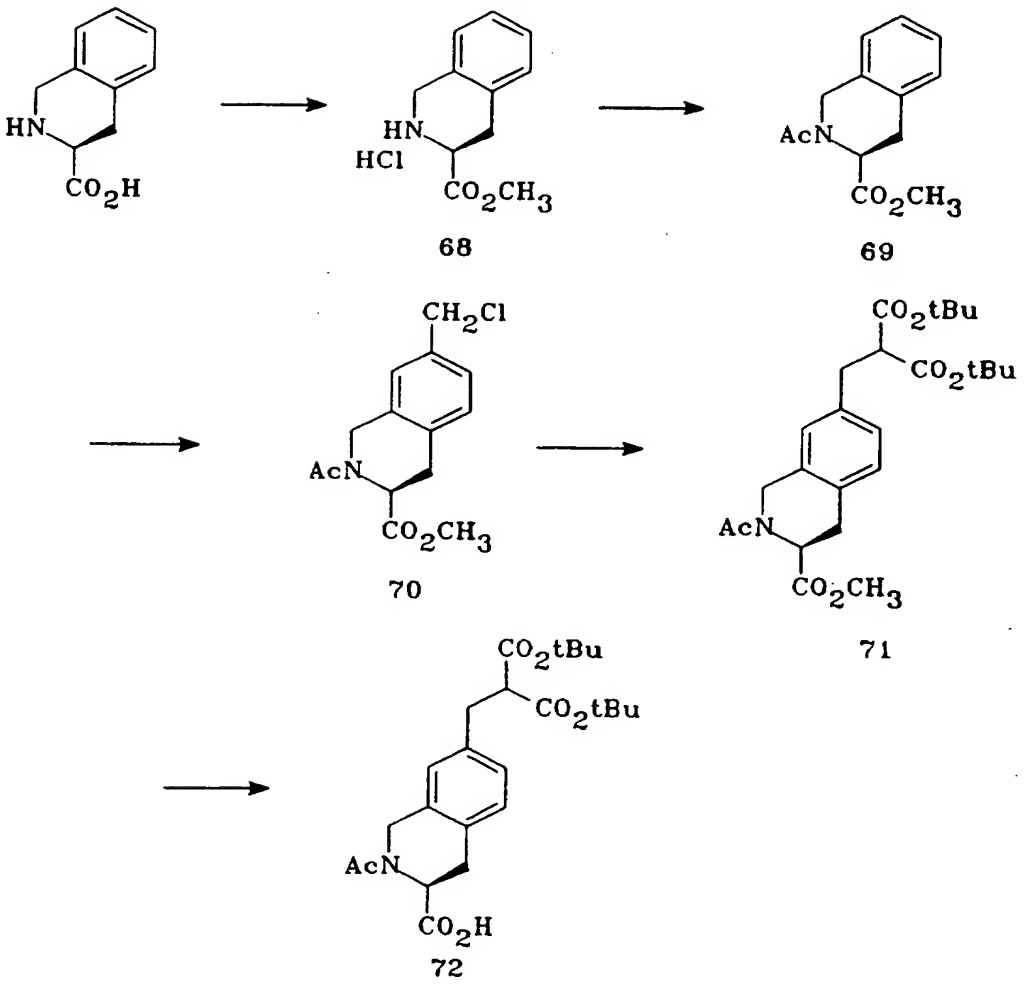


FIGURE 29

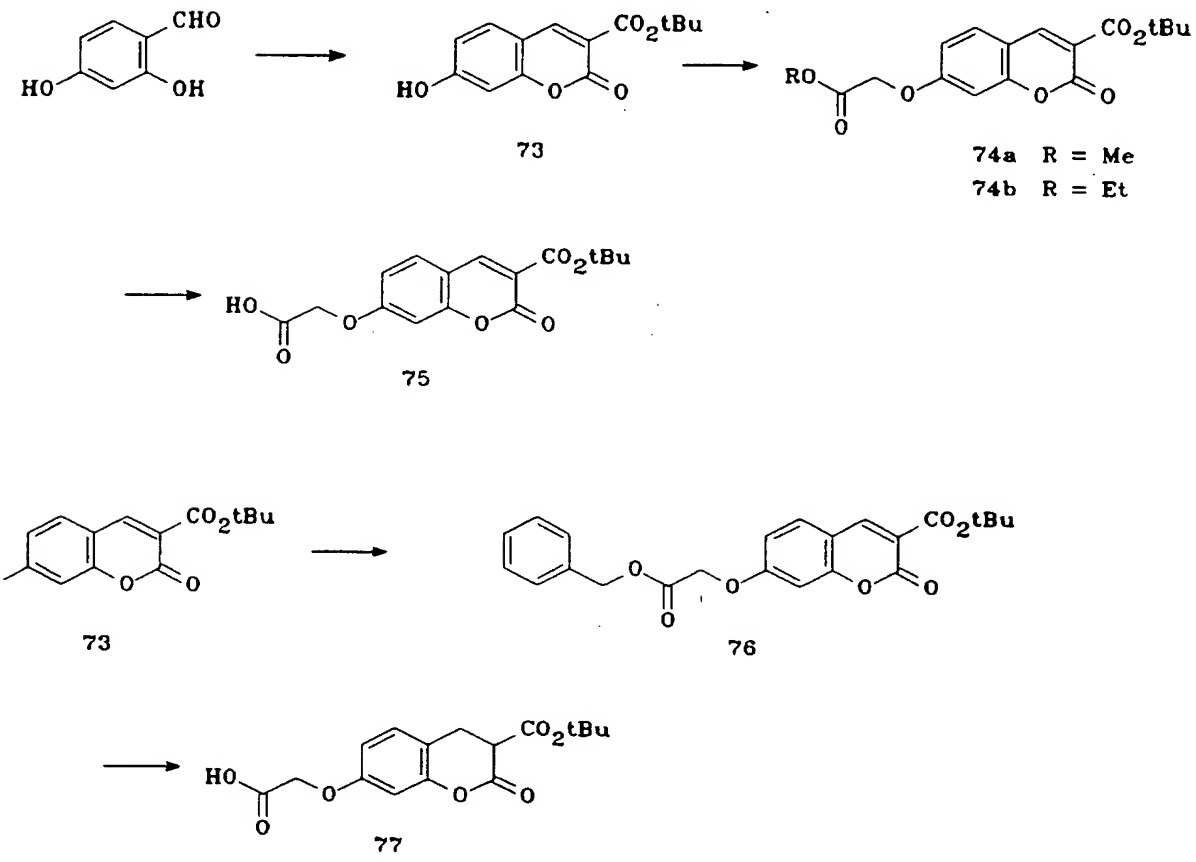


FIGURE 30

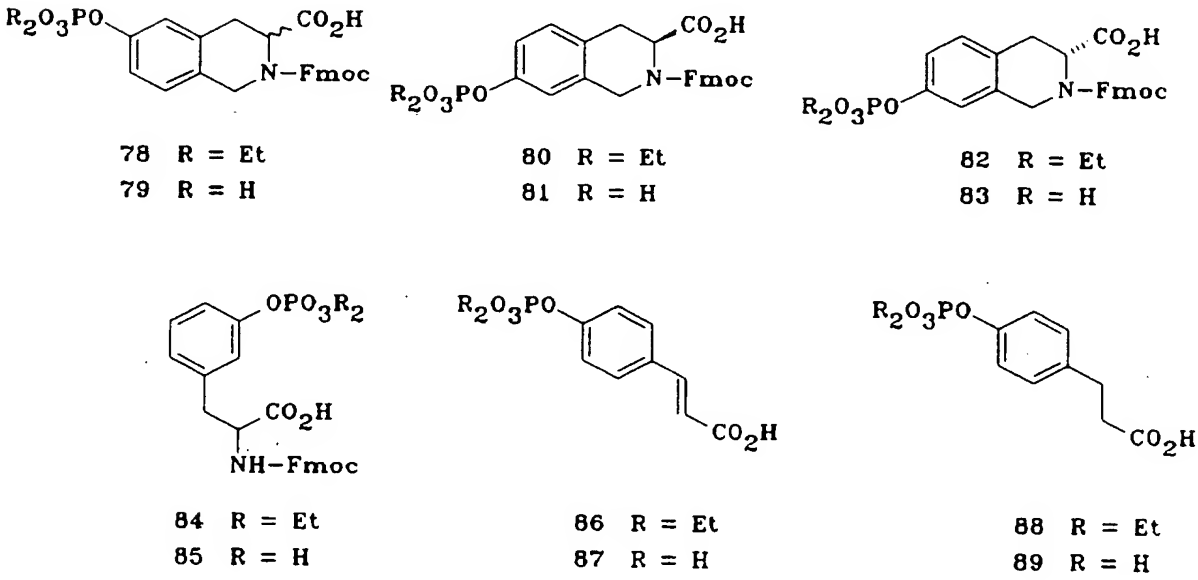


FIGURE 31

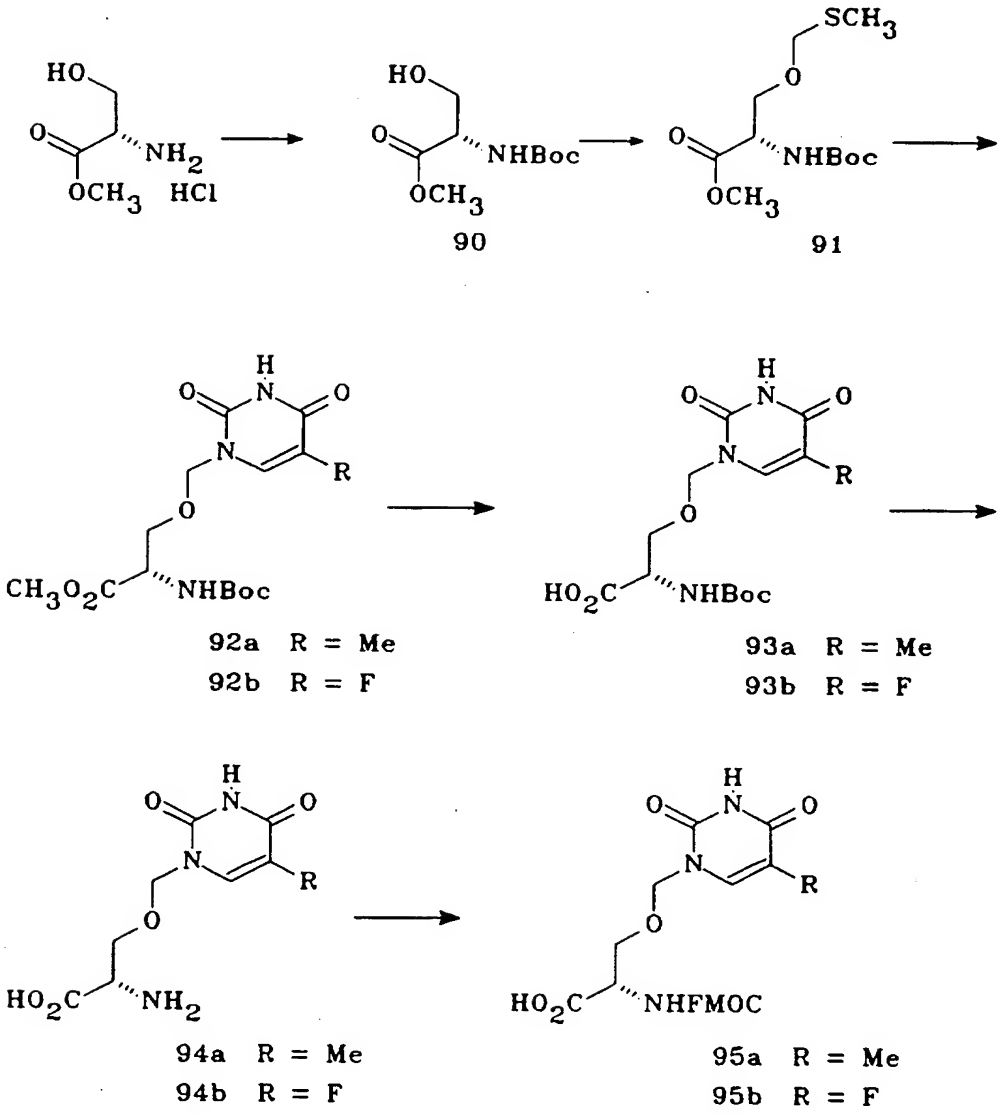


FIGURE 32

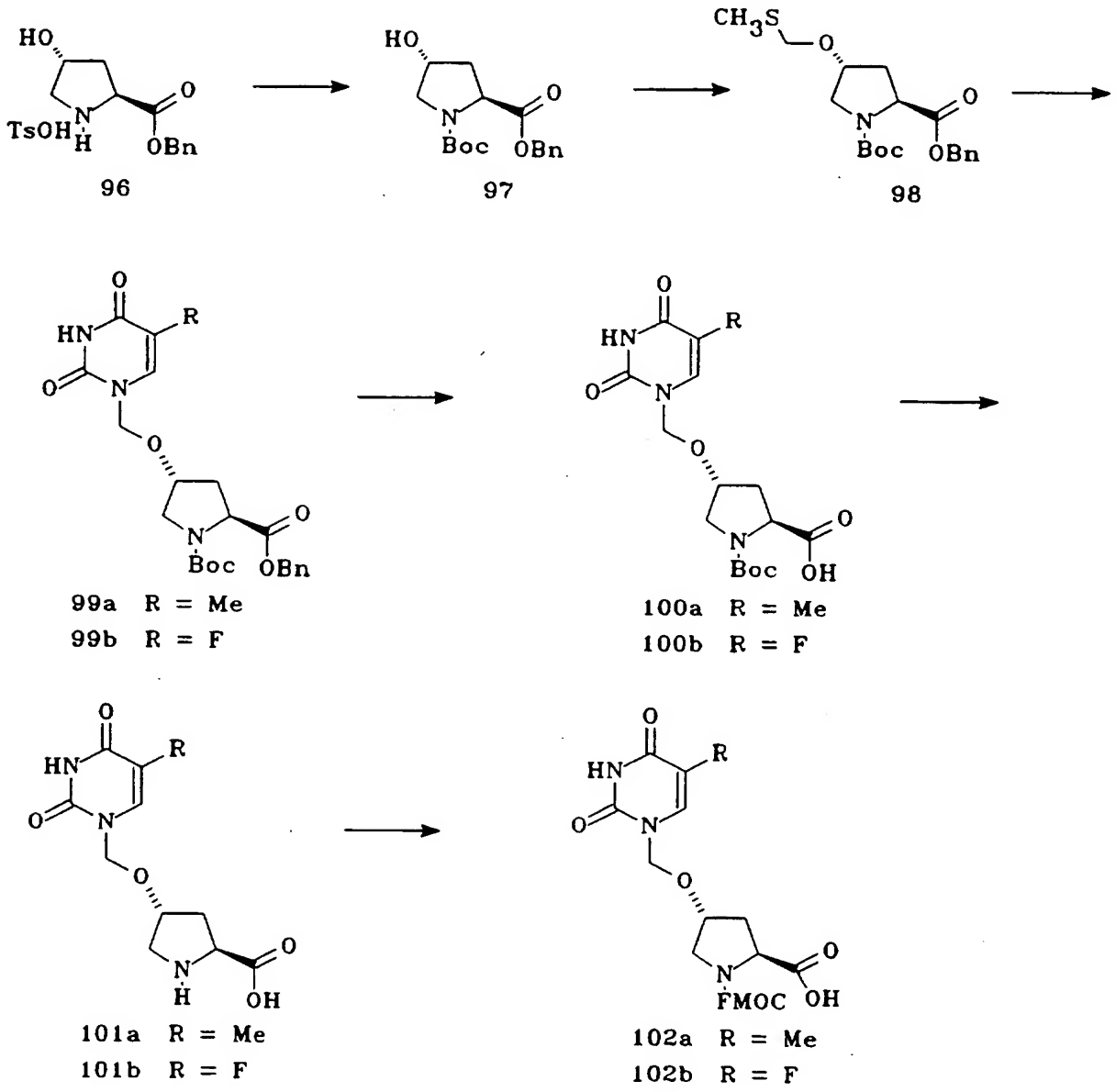


FIGURE 33

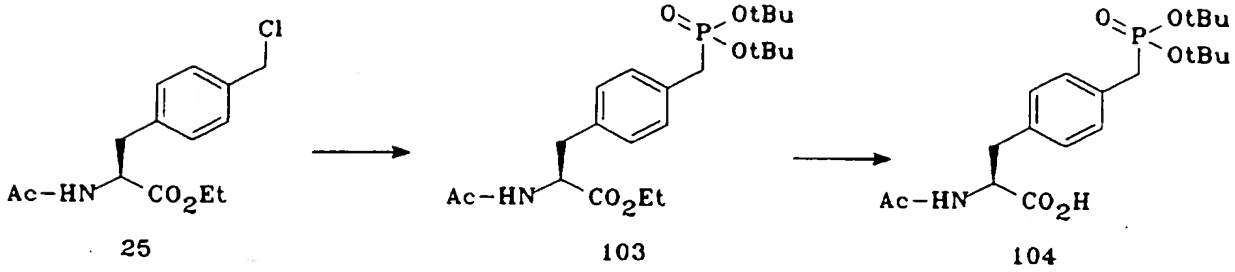


FIGURE 34

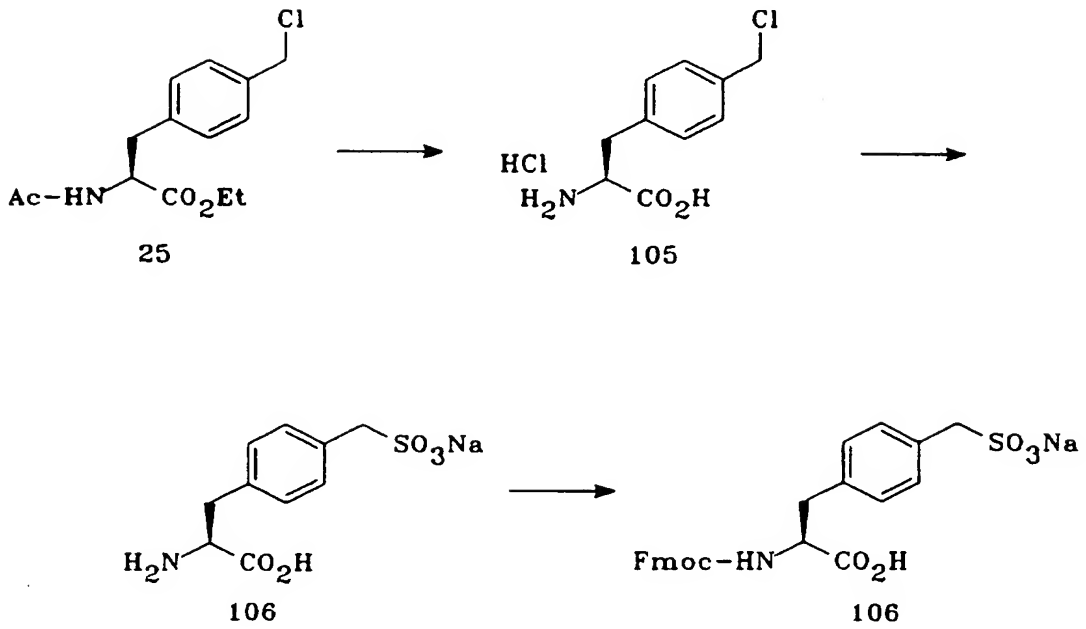


FIGURE 35

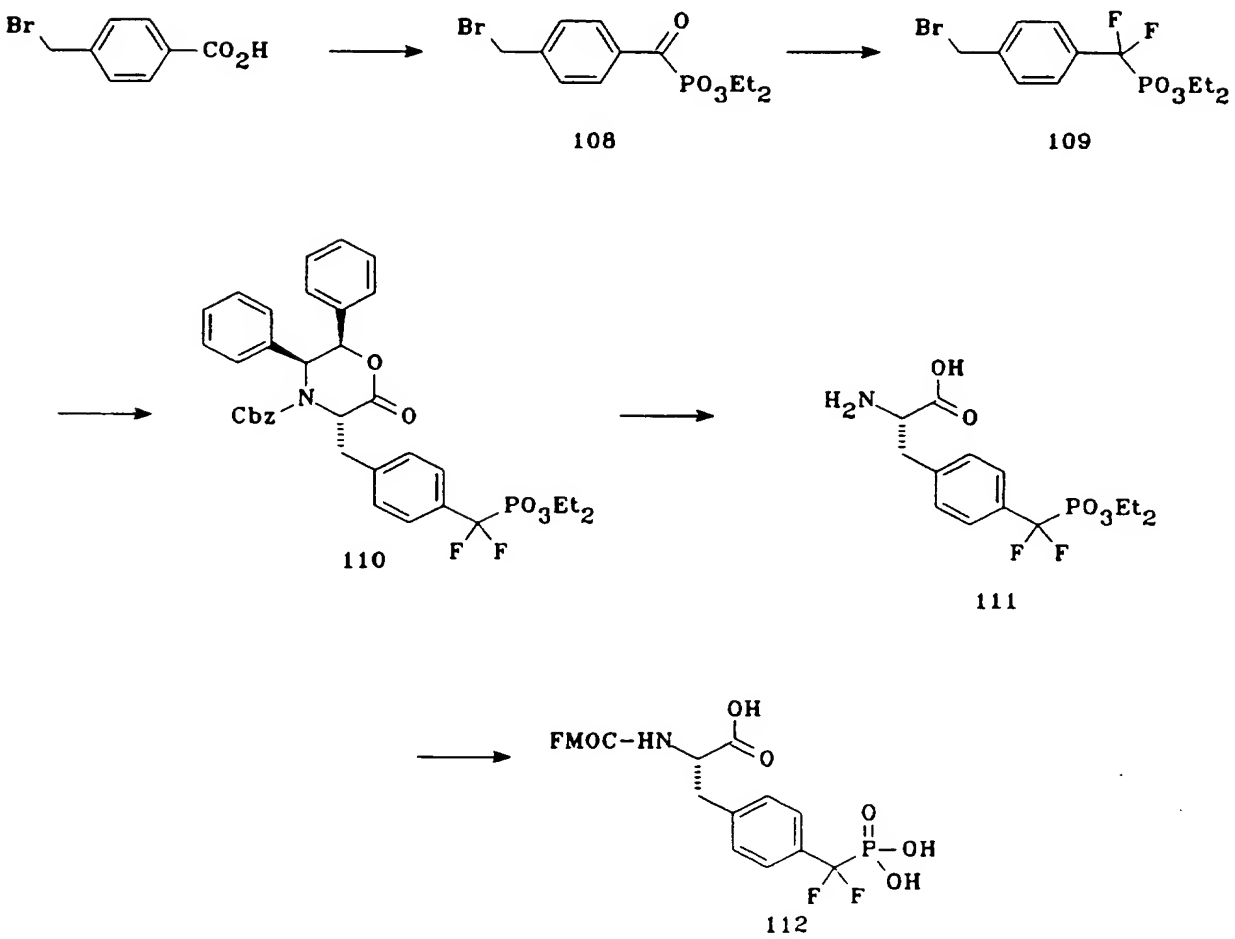


FIGURE 36

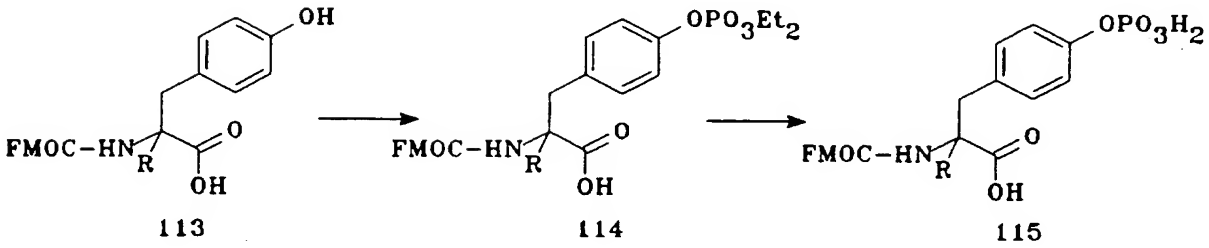


FIGURE 37

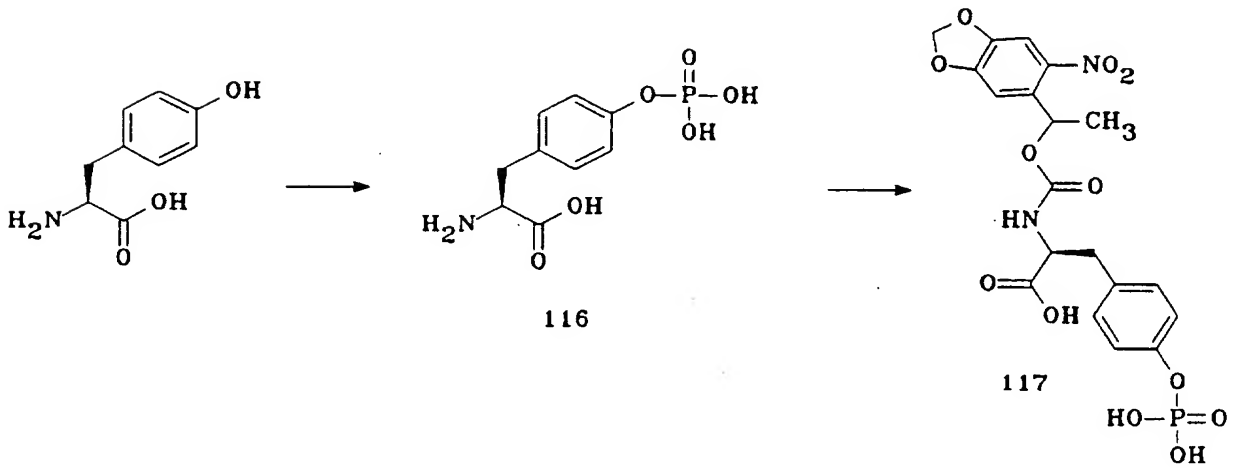


FIGURE 38 (a)

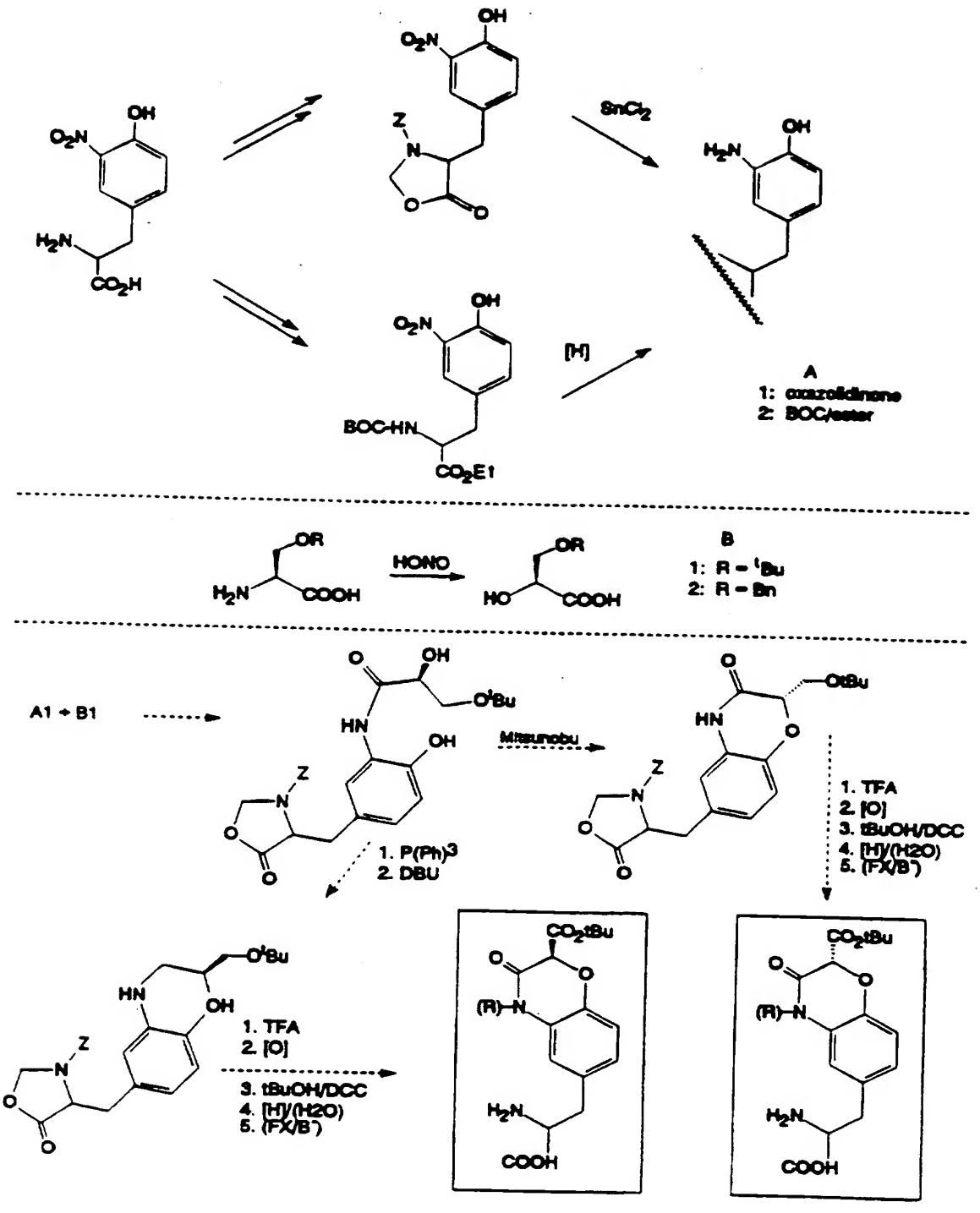


FIGURE 38 (b)

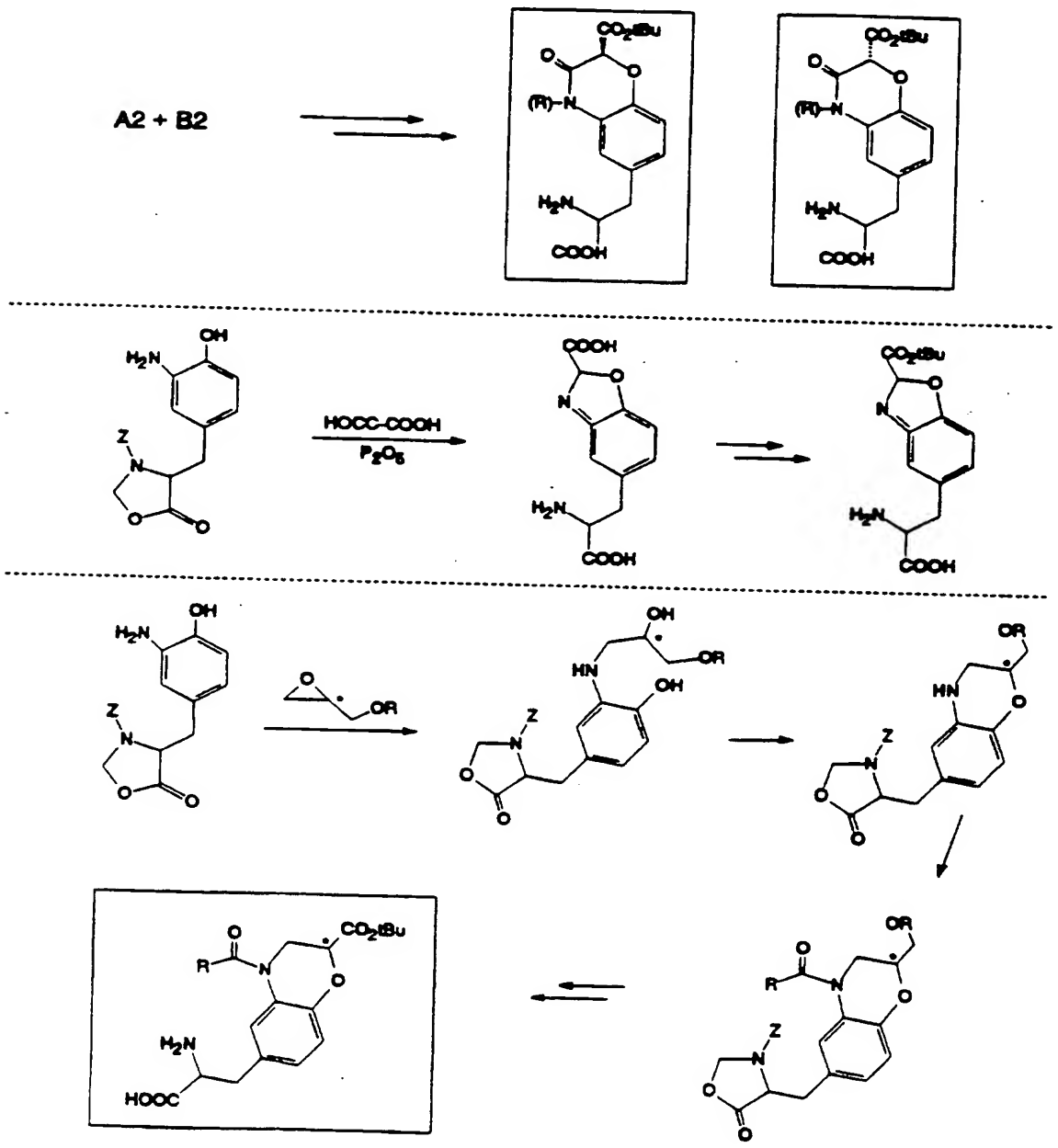


FIGURE 38 (c)

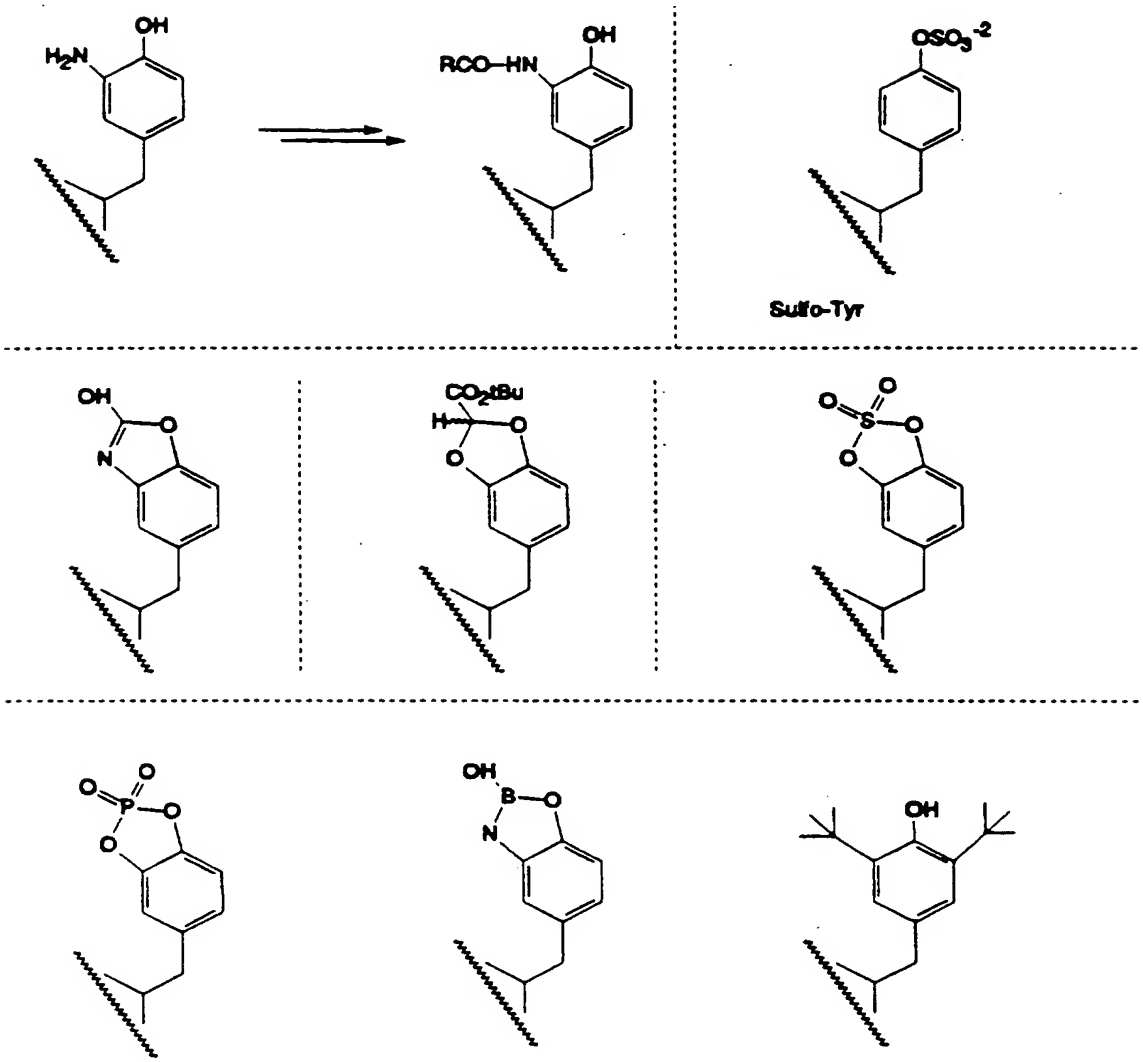


FIGURE 38 (d)

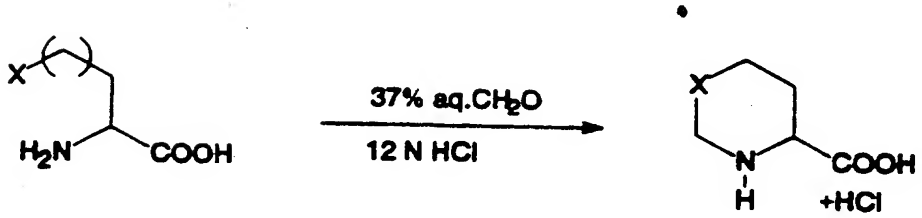


FIGURE 38 (e)

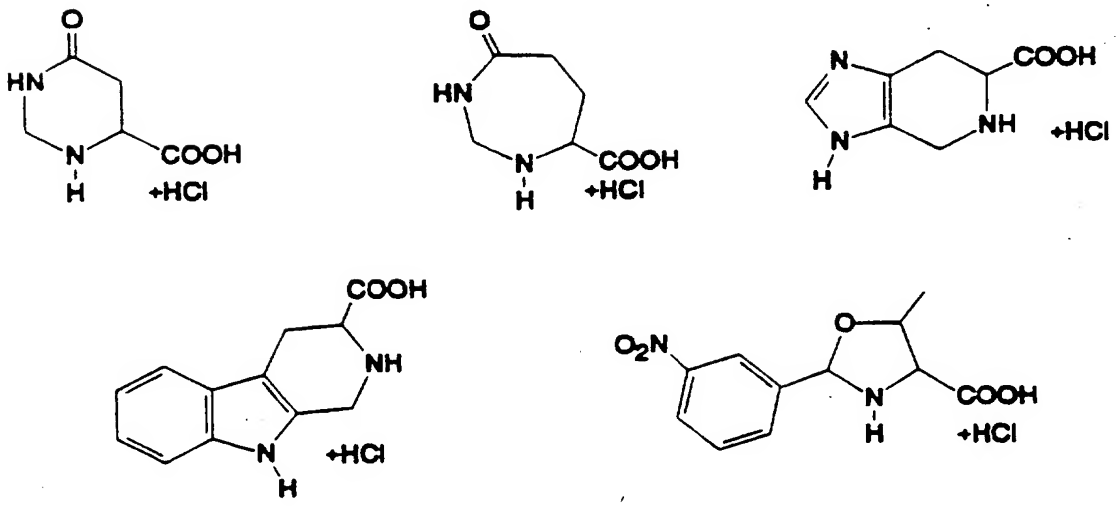


FIGURE 39

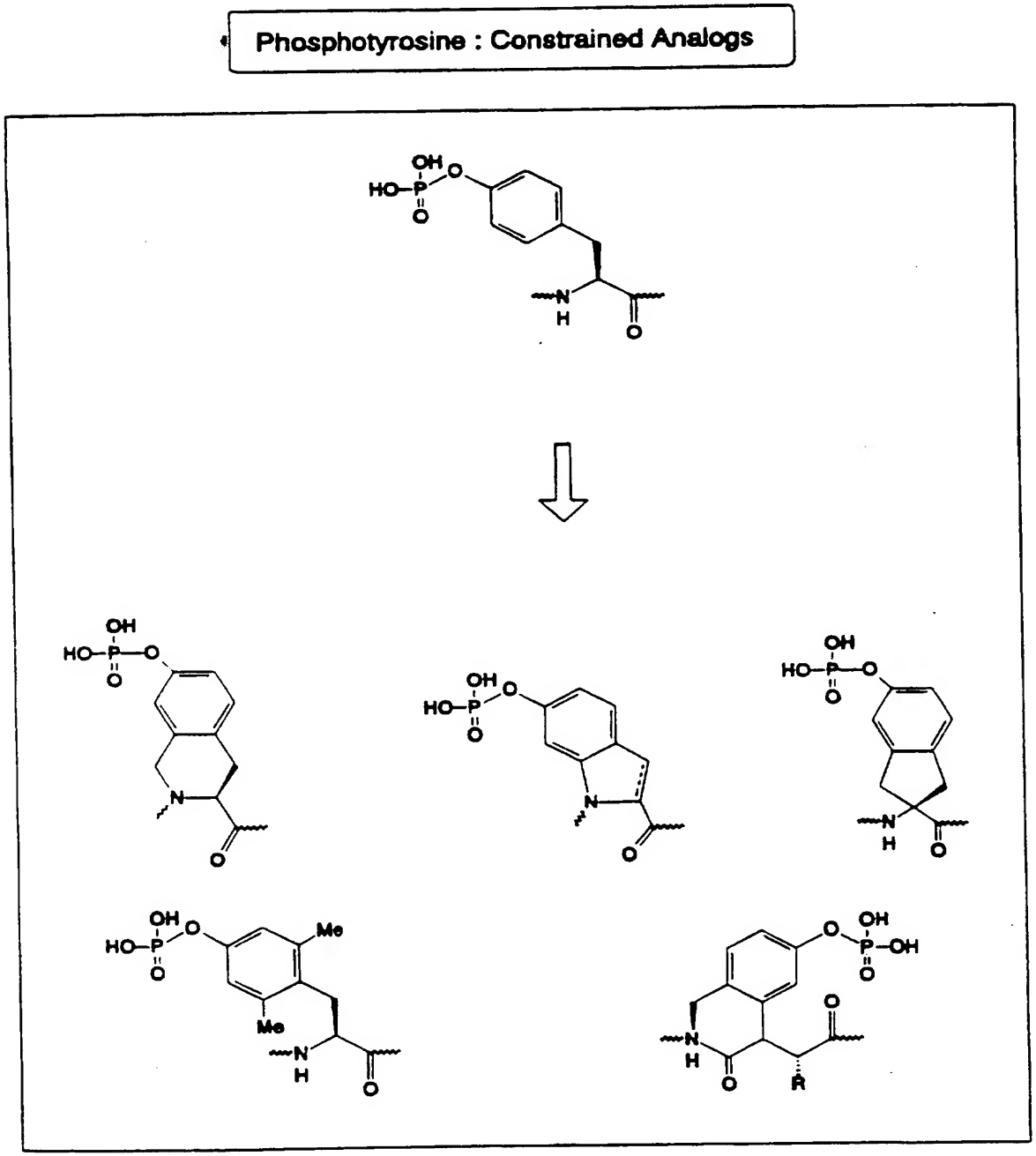


FIGURE 40

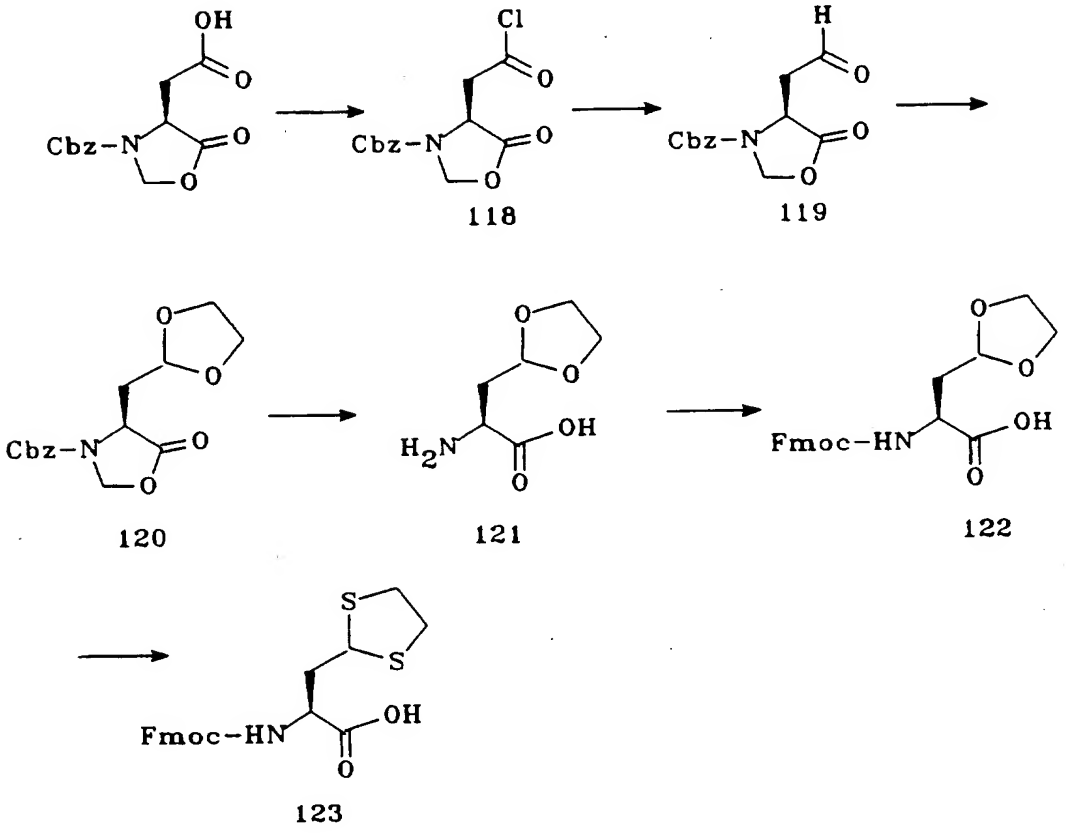


FIGURE 41

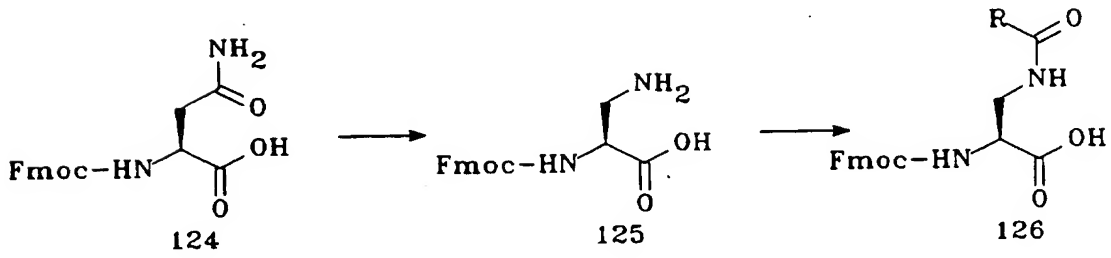


FIGURE 42

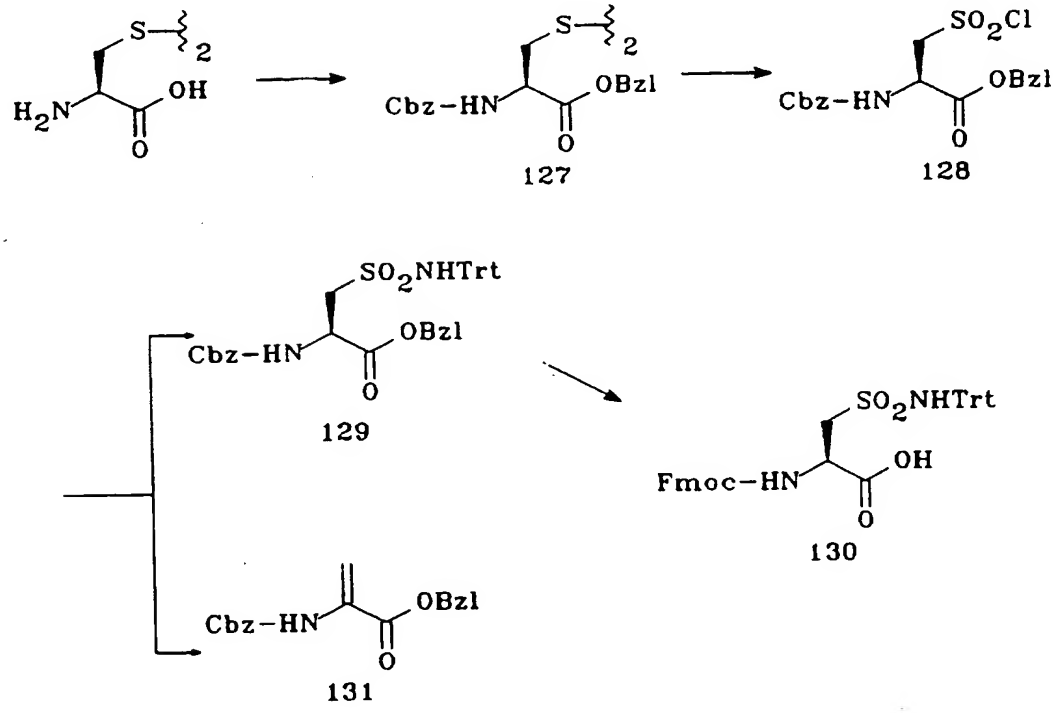


FIGURE 43

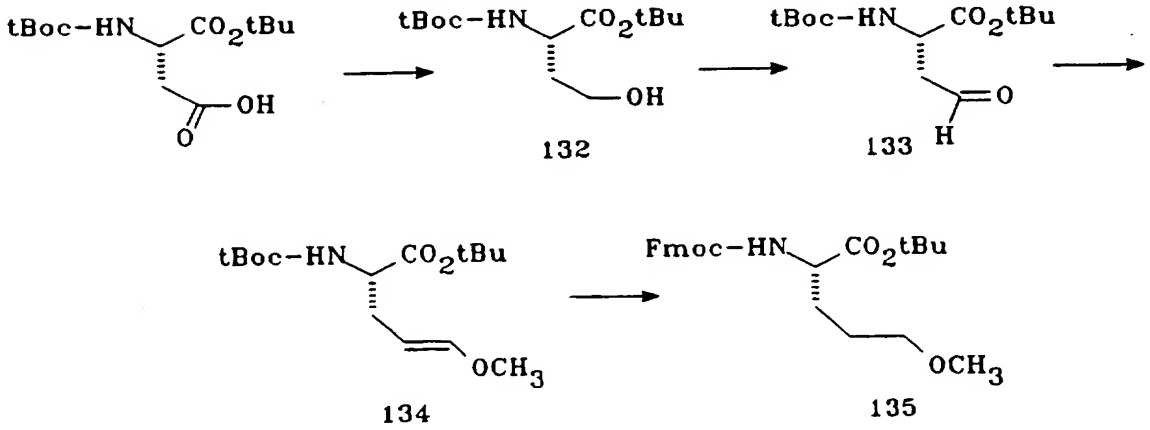


FIGURE 44

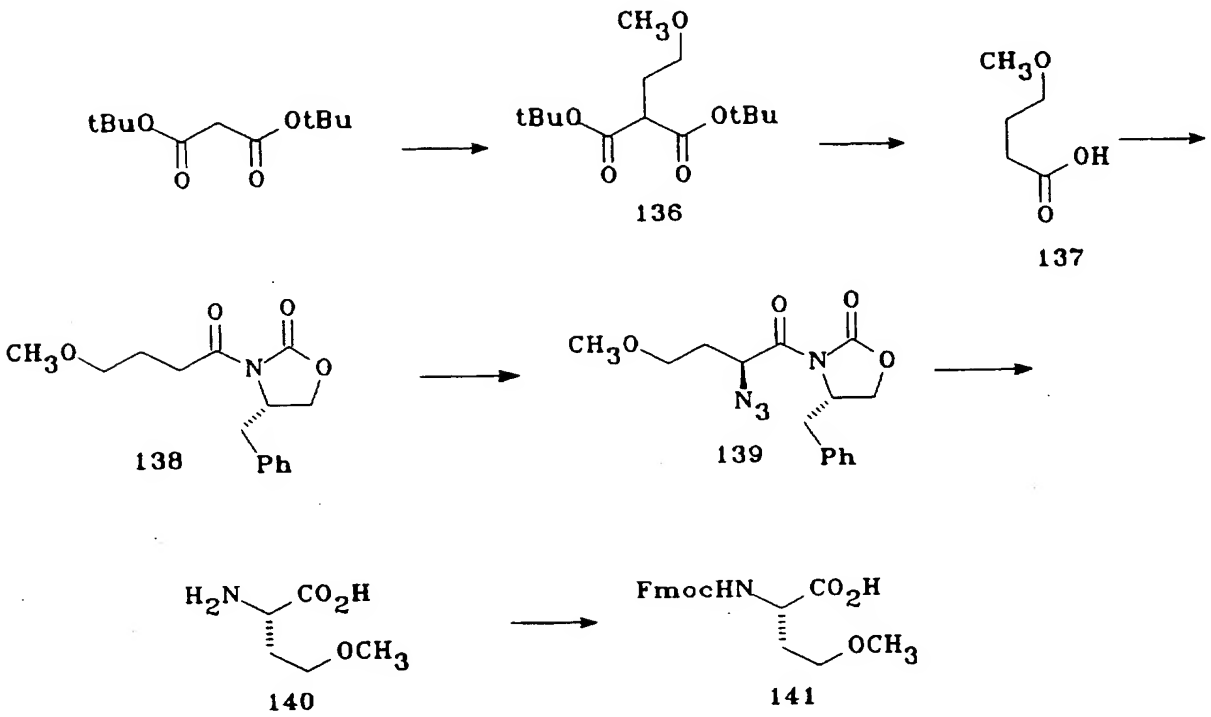


FIGURE 45

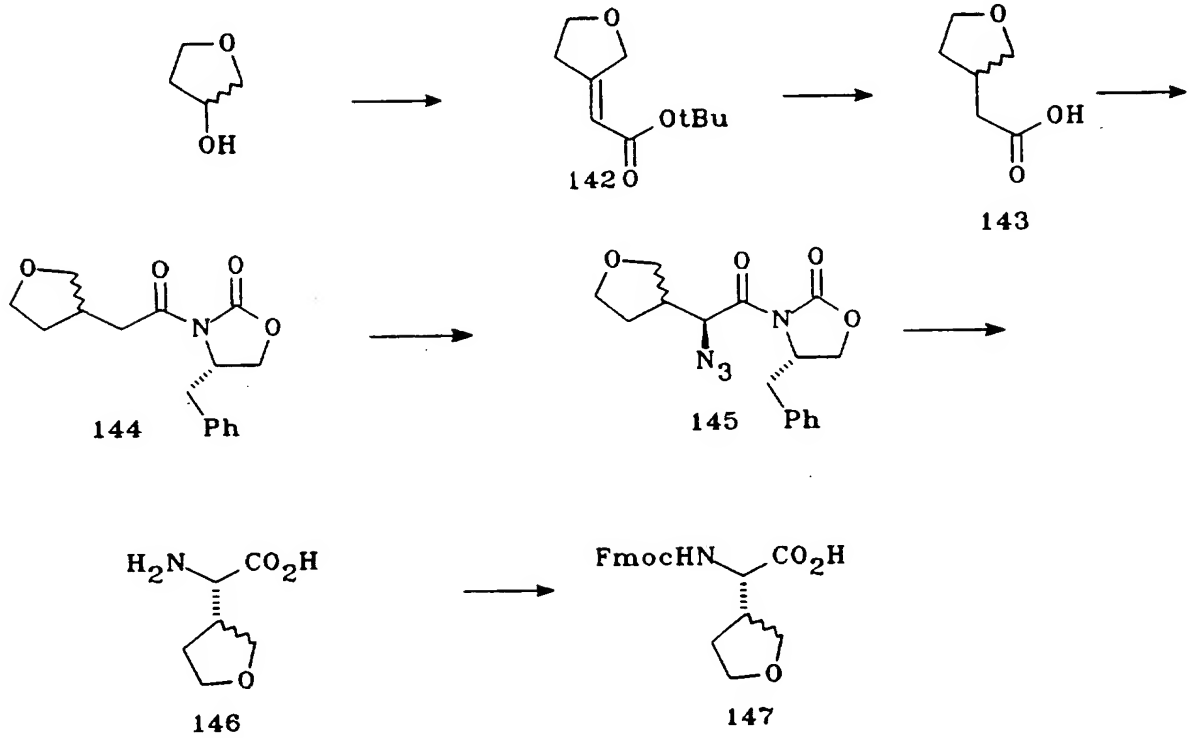
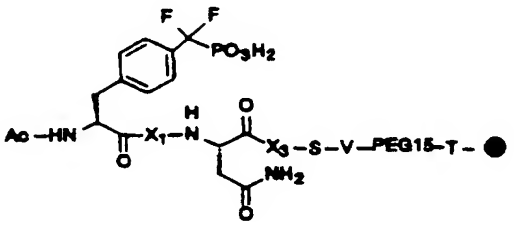
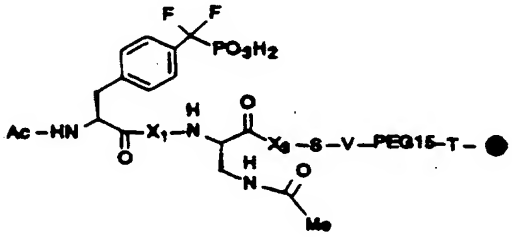


FIGURE 46

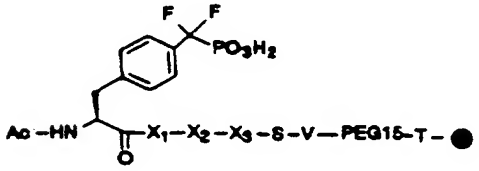
Combinatorial Libraries with Unnatural Amino Acids and F_2Pmp



Fmoc 624 M/g



Fmoc 623 M/g



Fmoc 626 M/g

X_1, X_2, and X_3 are 34 unnatural amino acids (shown on the following page), Asp and Glu

FIGURE 47(a)

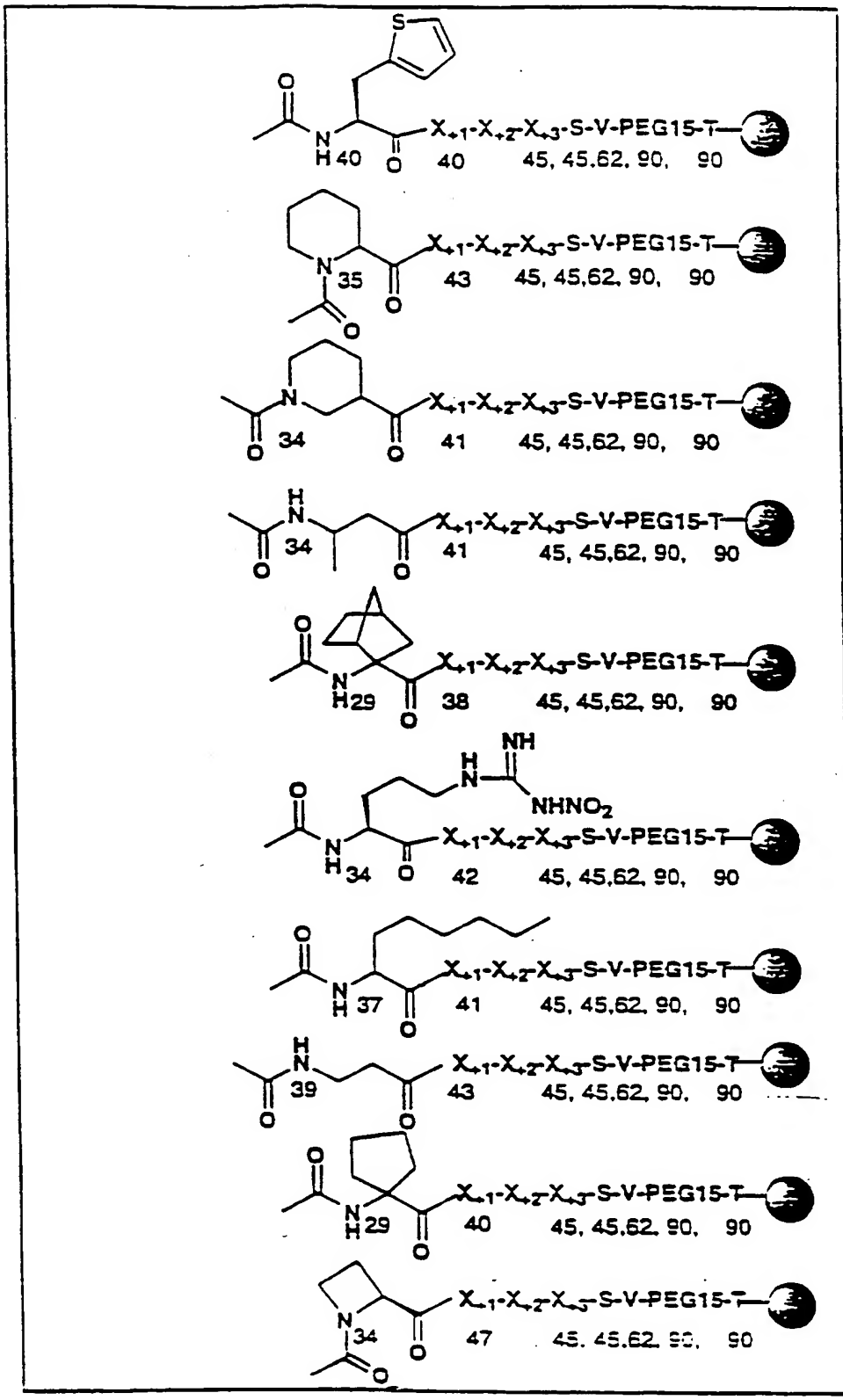


FIGURE 47(b)

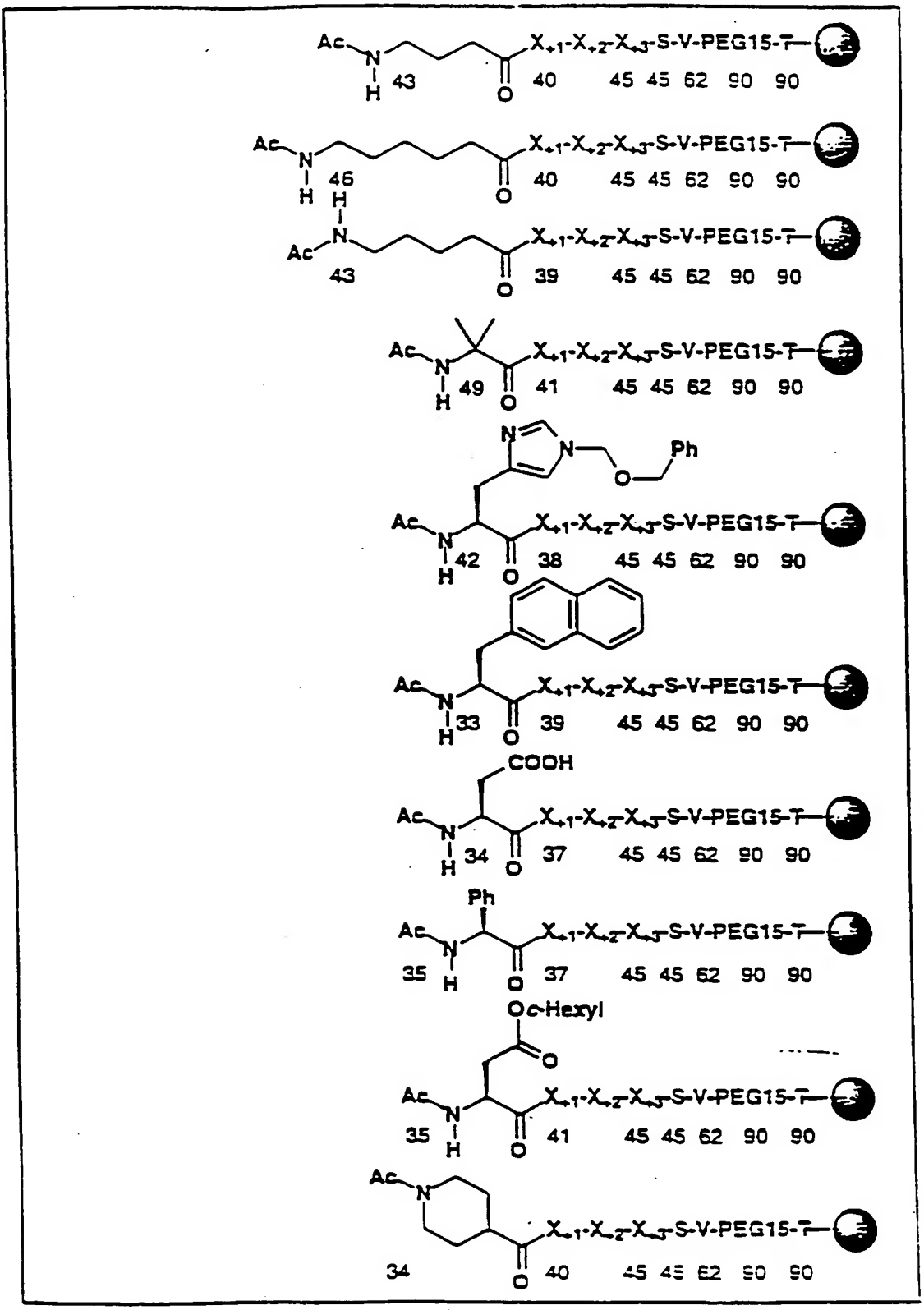


FIGURE 47(c)

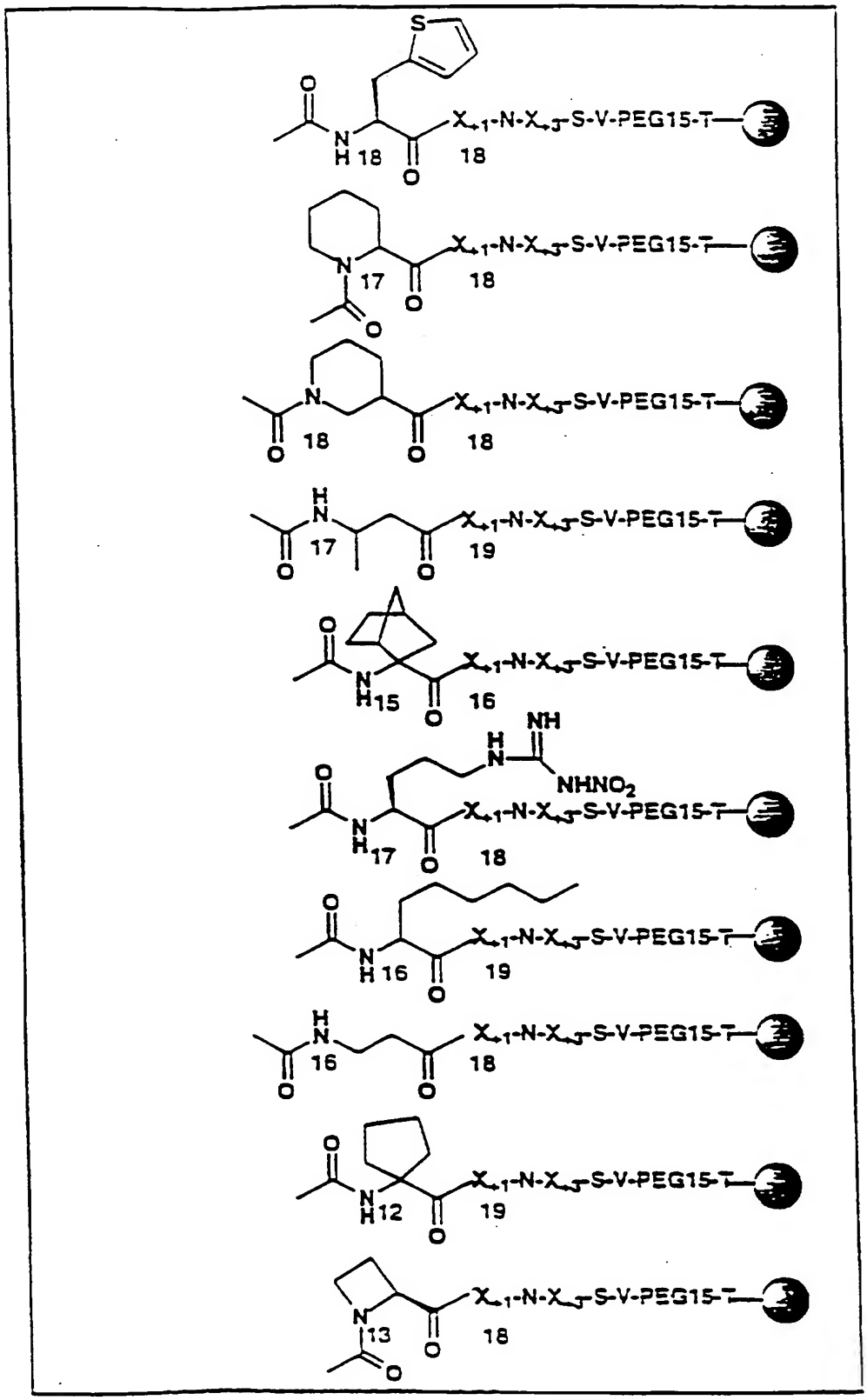


FIGURE 47(d)

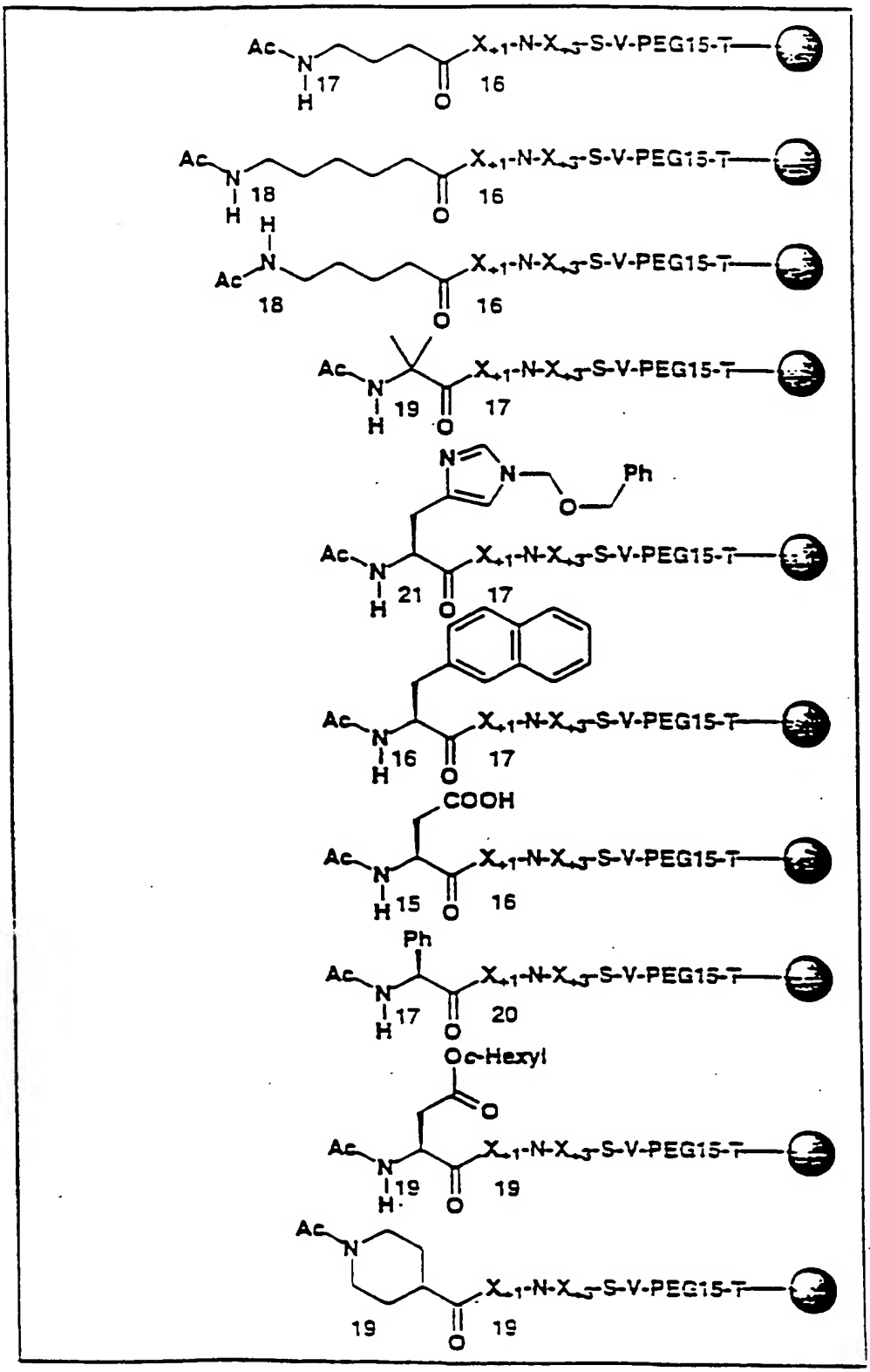
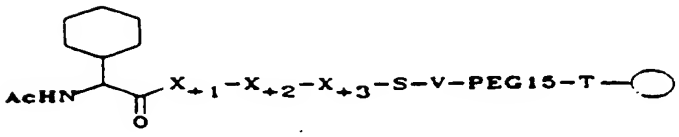
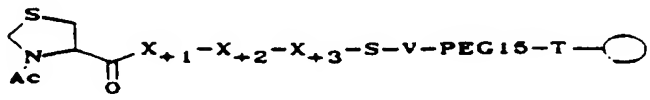
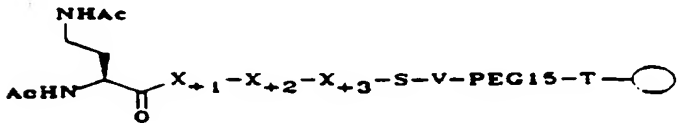
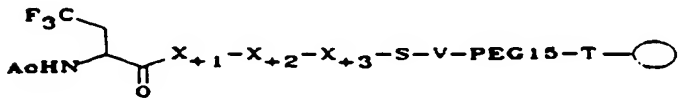


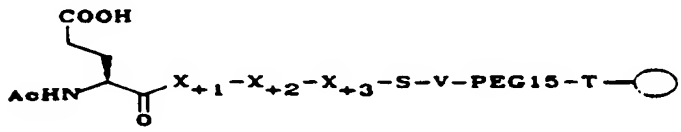
FIGURE 47(e)

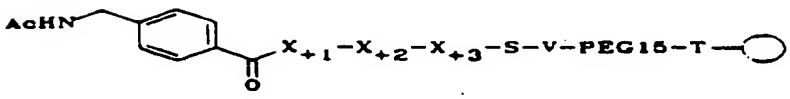

AcHN
X+1-X+2-X+3-S-V-PEG15-T

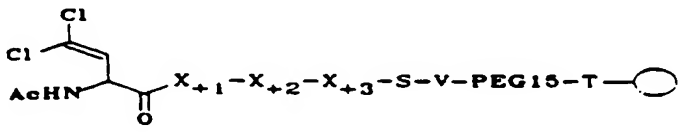

S
N
Ac
X+1-X+2-X+3-S-V-PEG15-T

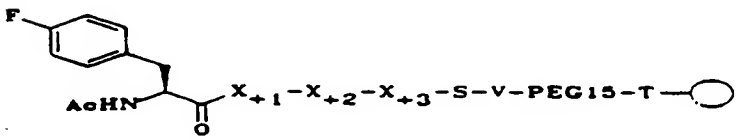

NHAc
AcHN
X+1-X+2-X+3-S-V-PEG15-T

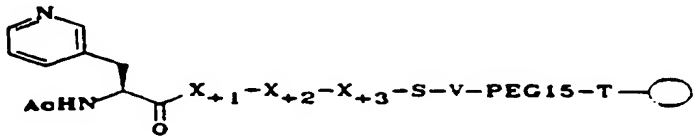

F3C
AcHN
X+1-X+2-X+3-S-V-PEG15-T


COOH
AcHN
X+1-X+2-X+3-S-V-PEG15-T


AcHN
X+1-X+2-X+3-S-V-PEG15-T


Cl
Cl
AcHN
X+1-X+2-X+3-S-V-PEG15-T


F
AcHN
X+1-X+2-X+3-S-V-PEG15-T


N
AcHN
X+1-X+2-X+3-S-V-PEG15-T

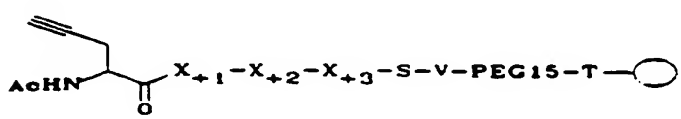
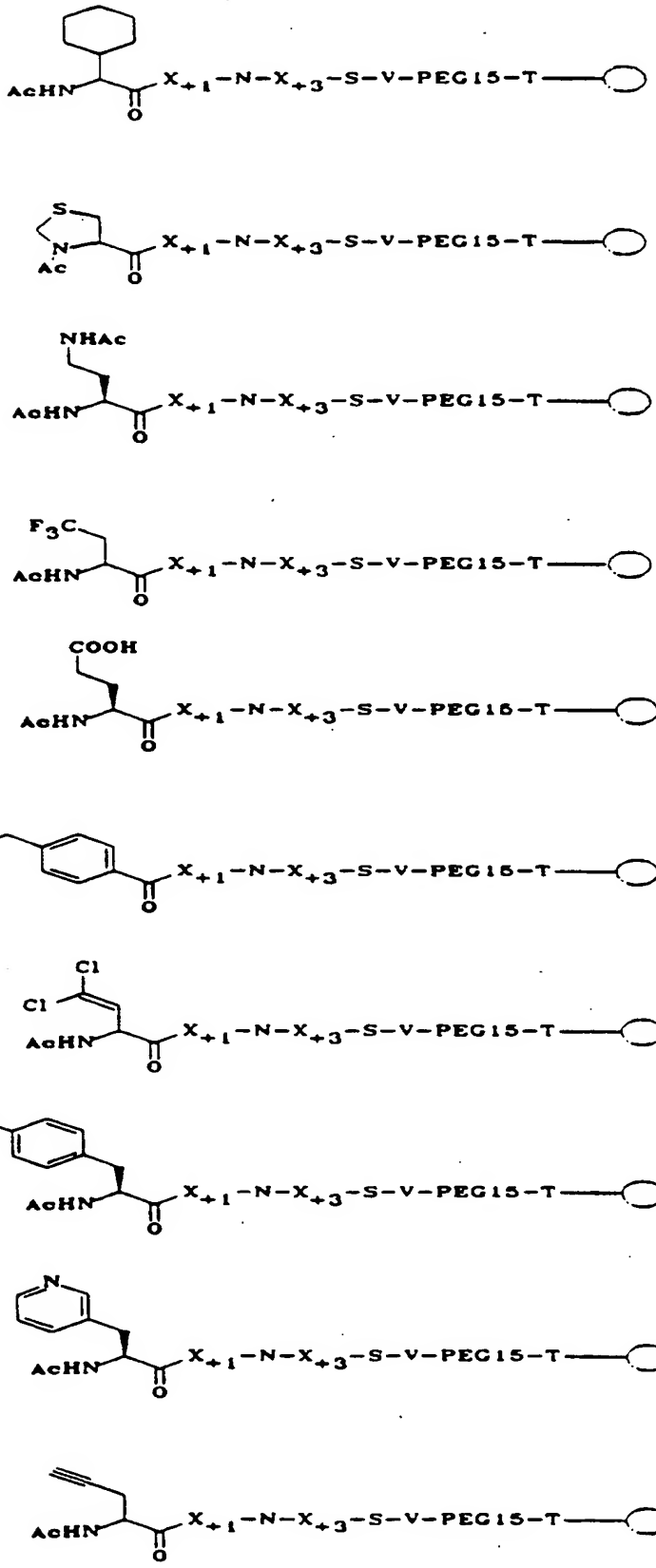

AcHN
X+1-X+2-X+3-S-V-PEG15-T

FIGURE 47(f)



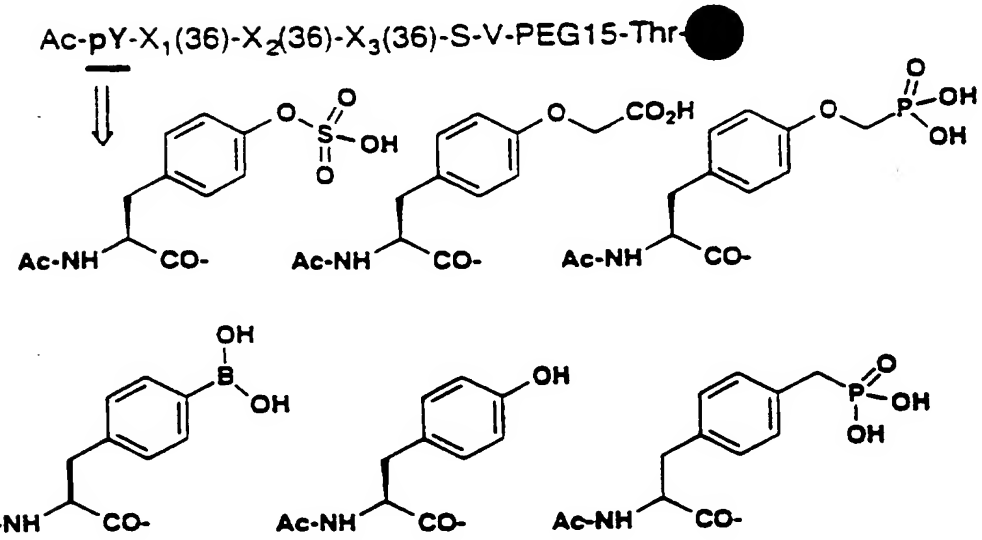


FIGURE 48(a)

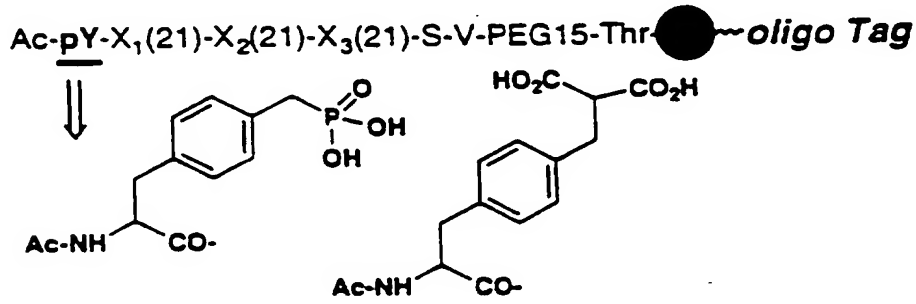


FIGURE 48(b)

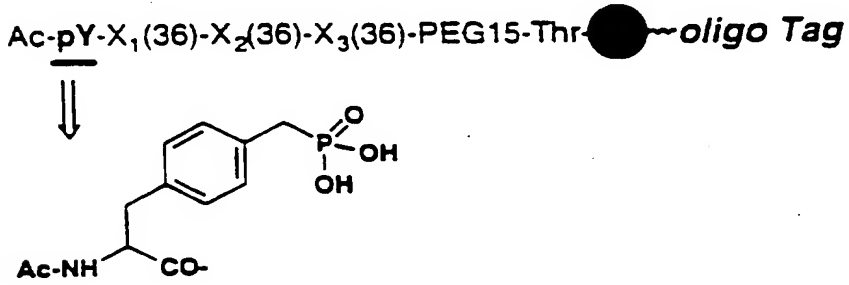


FIGURE 48(c)

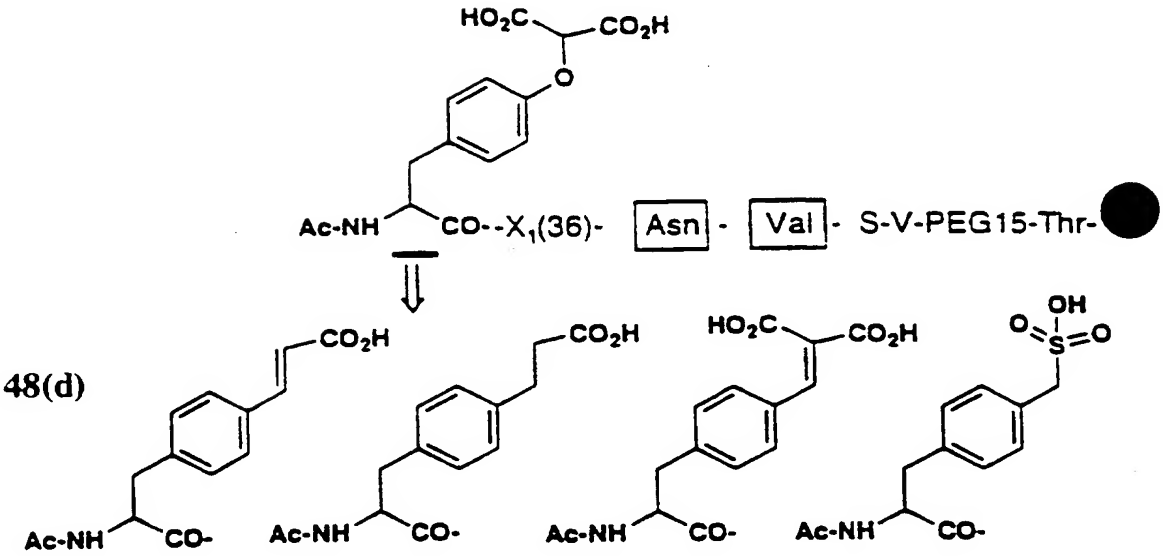


FIGURE 48(d)

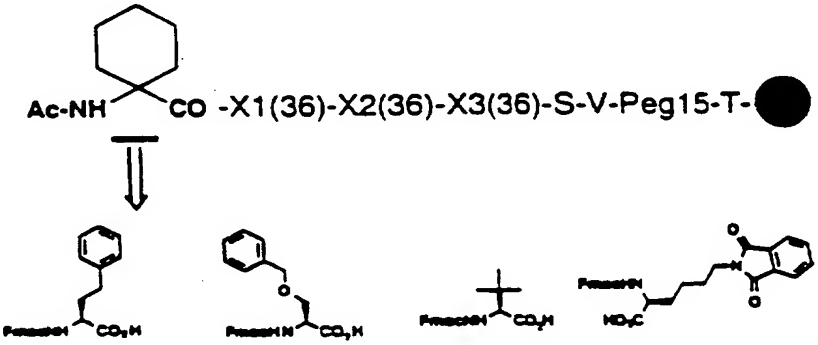


FIGURE 48(e)

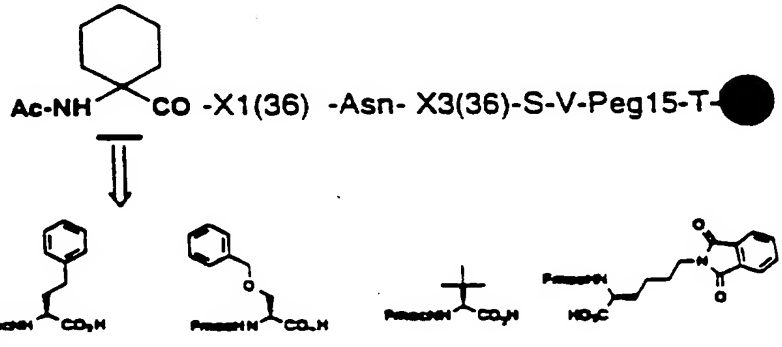


FIGURE 48(f)

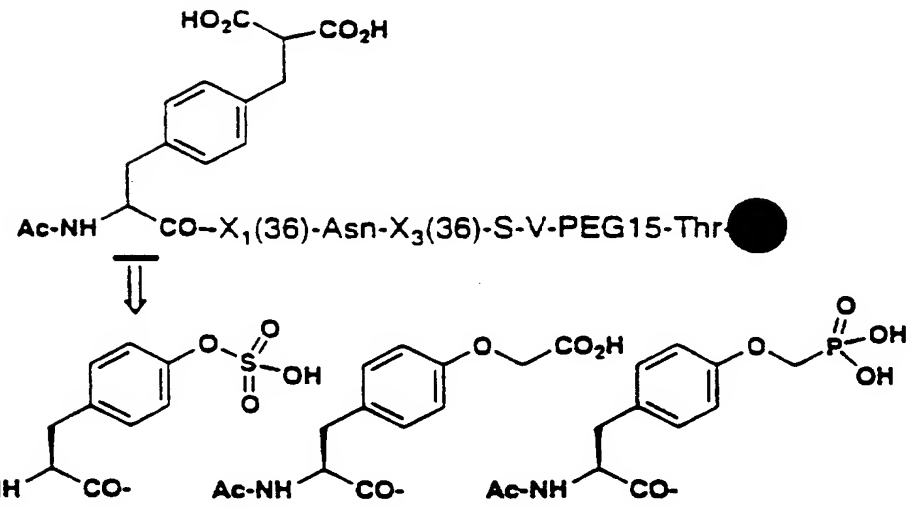
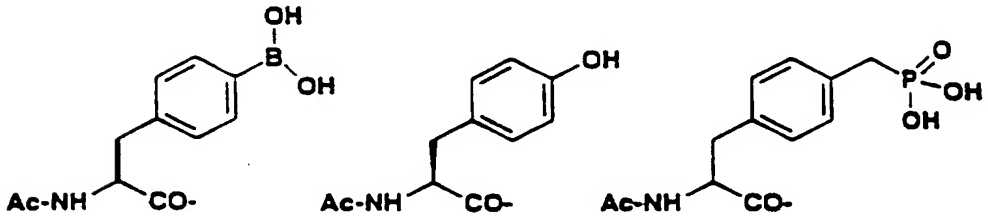


FIGURE 48(g)



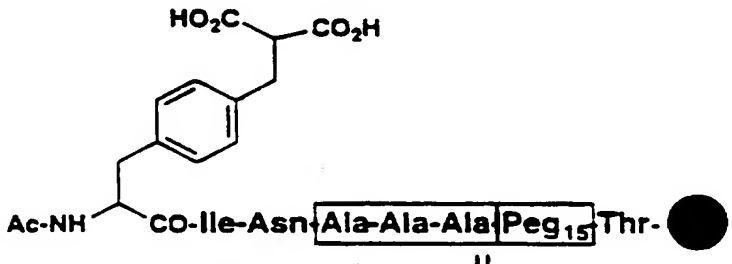


FIGURE 48(h)

-Gln-Ala-Ala-Peg_{15}-

-Peg_{15}-Peg_{15}-

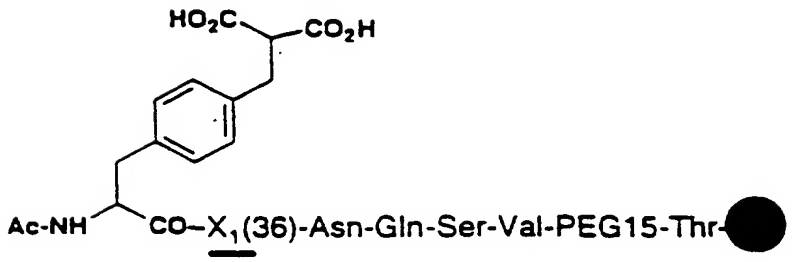
-Gln-Ser-Val-Peg_{15}-

-Gln-Peg_{15}-Peg_{15}-

-Gln-Peg_{15}-

-Peg_{15}-

FIGURE 48(i)



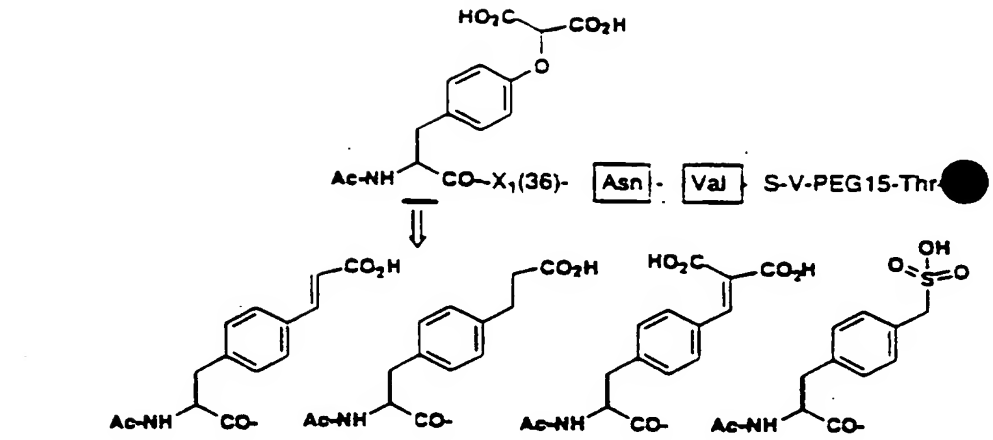
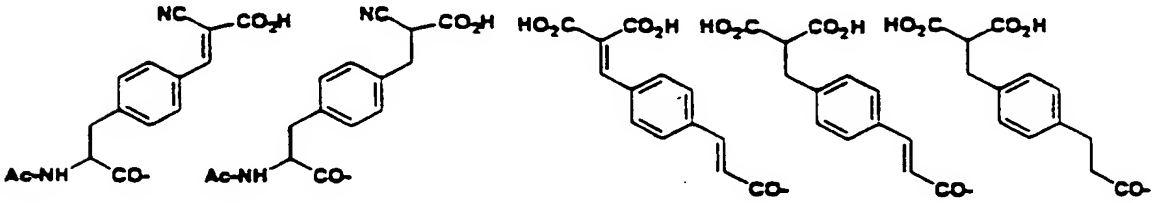


FIGURE 48(j)



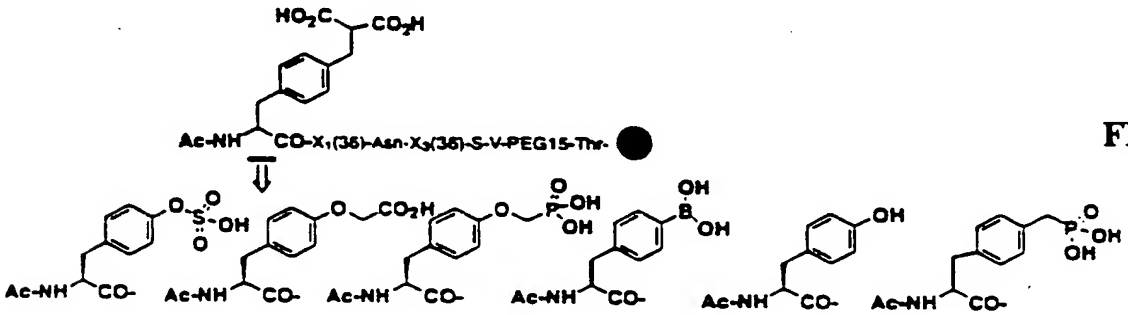


FIGURE 48(k)

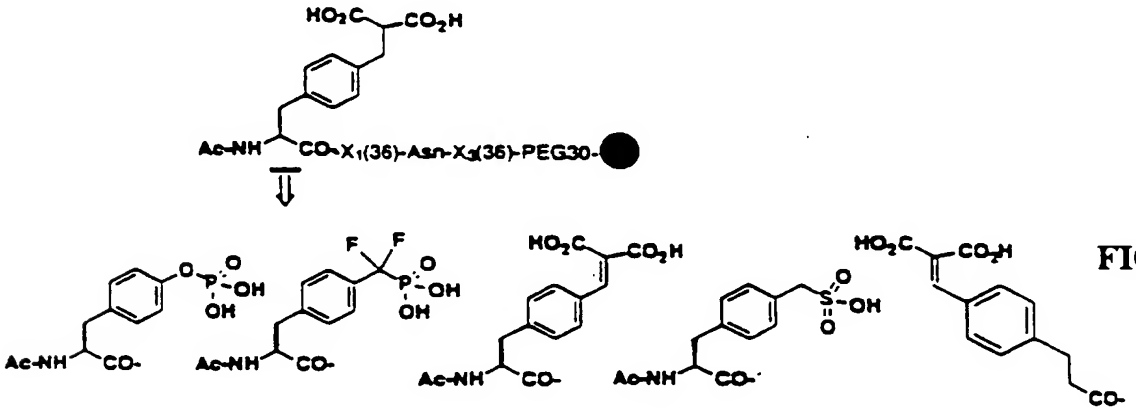


FIGURE 48(l)

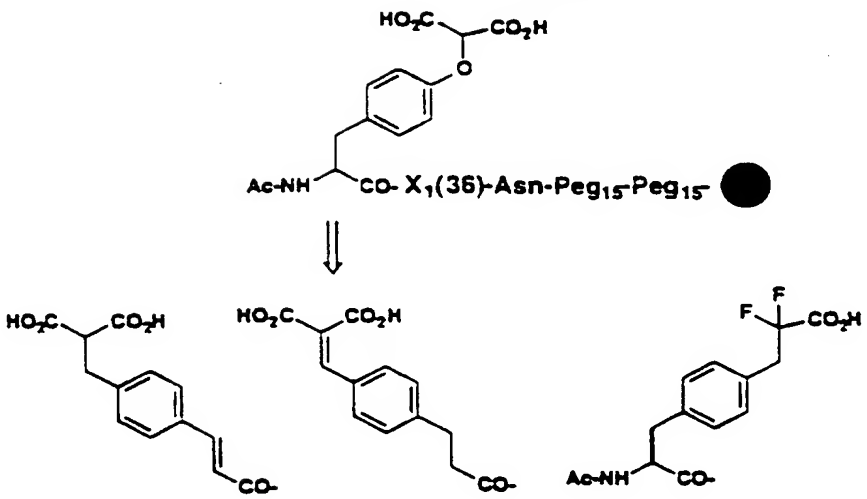
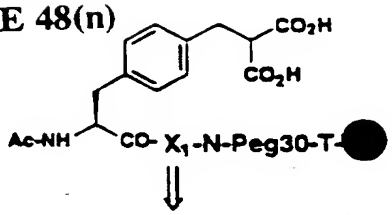


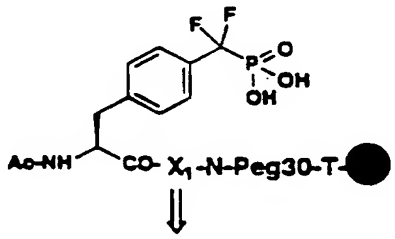
FIGURE 48(m)

FIGURE 48(n)



: X_1= 19 aromatic
: 8 basic
: 7 acidic
: 17 backbone
: 23 H-bond
: 17 hydrophobic
: 19 Lys der.
: 19 d-AAs
: 19 L-AAs

FIGURE 48(o)



: X_1= 19 aromatic
: 8 basic
: 7 acidic
: 17 backbone
: 23 H-bond
: 17 hydrophobic
: 19 Lys der.
: 19 d-AAs
: 19 L-AAs

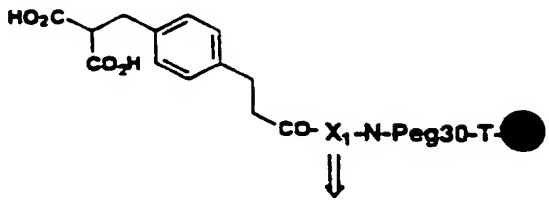
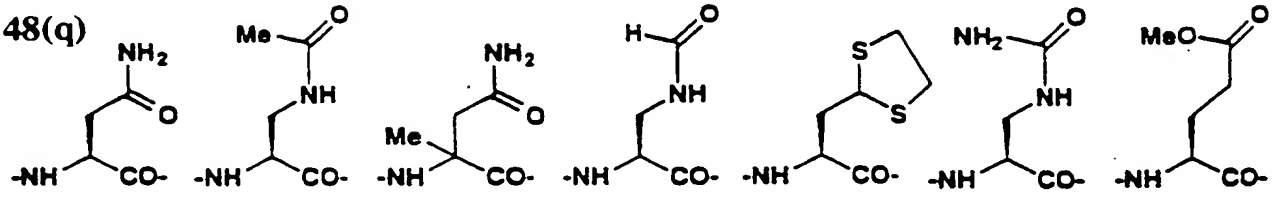


FIGURE 48(p)

: X_1= 19 aromatic
: 8 basic
: 7 acidic
: 17 backbone
: 23 H-bond
: 17 hydrophobic
: 19 Lys der.
: 19 d-AAs

Ac-pY-X_1(36)-Asn-I-X_3(36)-S-V-PEG15-Thr-●

FIGURE 48(q)



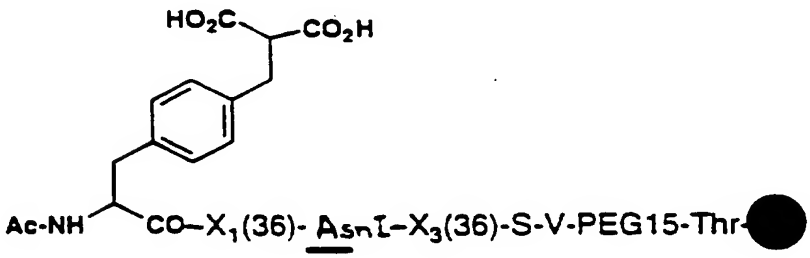
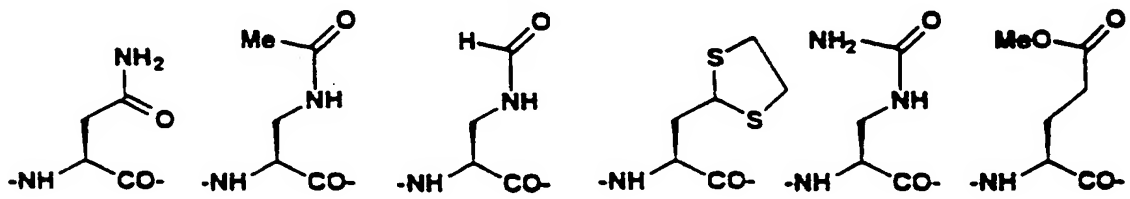


FIGURE 48(r)



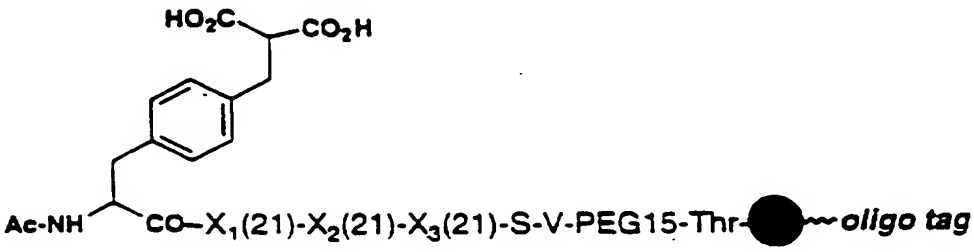
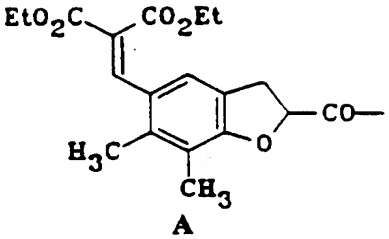
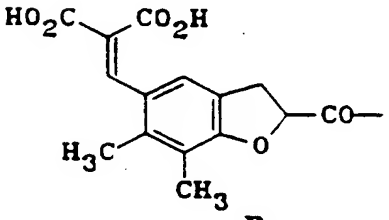


FIGURE 48(s)

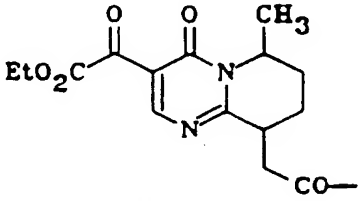
FIGURE 49 (1 of 4)



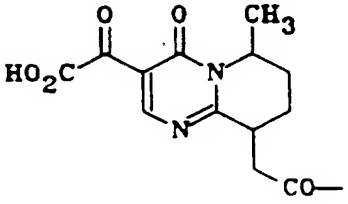
A



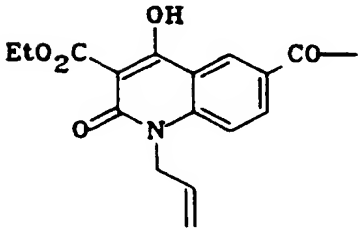
B



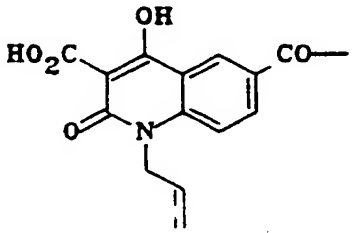
C



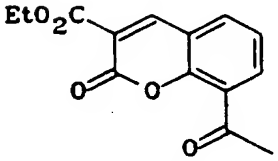
D



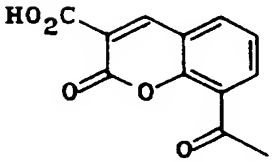
E



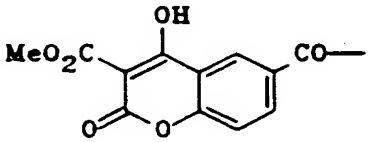
F



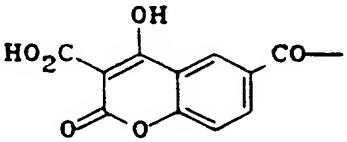
G



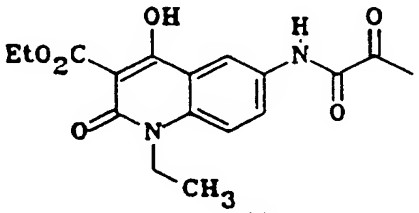
H



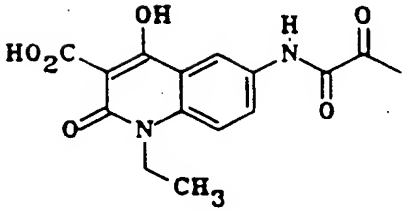
I



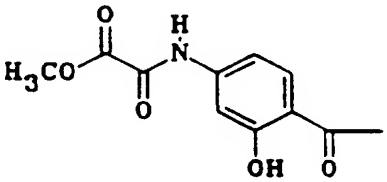
J



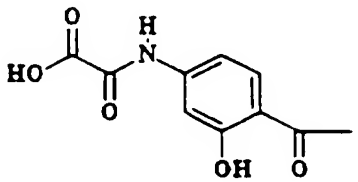
K



L

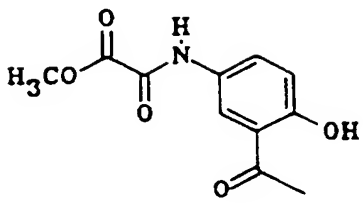


M

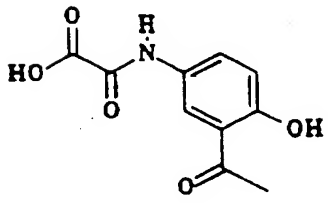


N

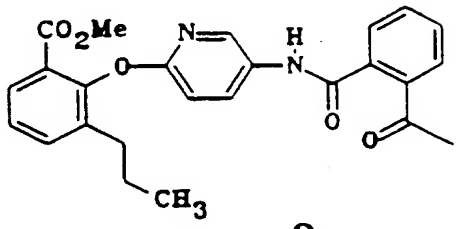
FIGURE 49 (2 of 4)



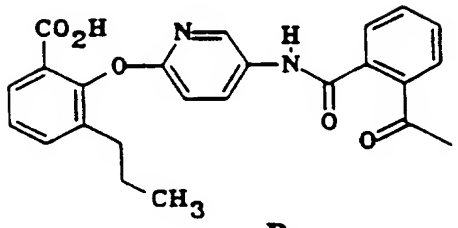
O



P



Q



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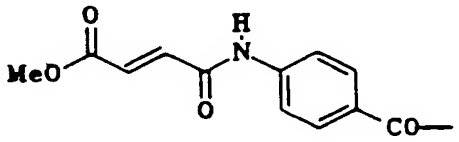

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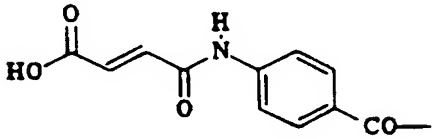

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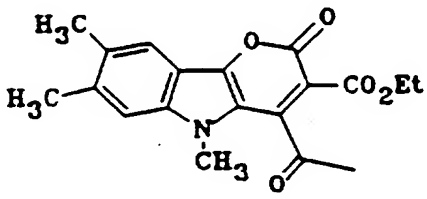

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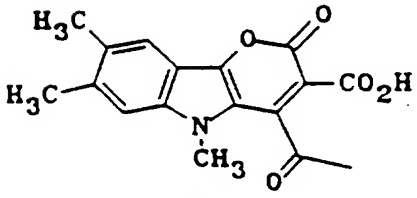

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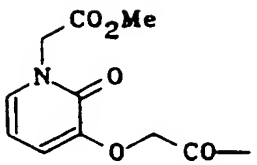

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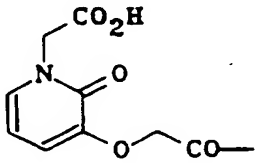

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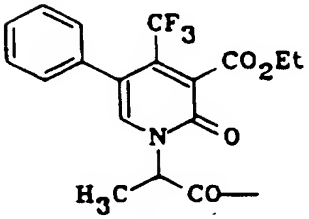

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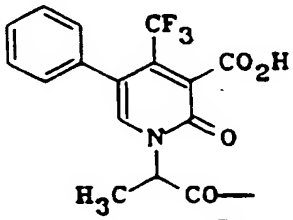

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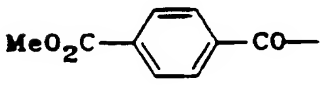
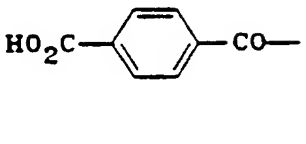


AA

FIGURE 49 (3 of 4)



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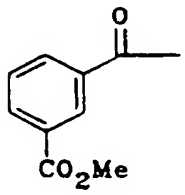

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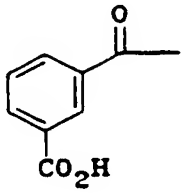

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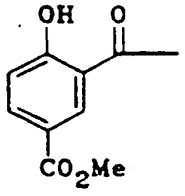

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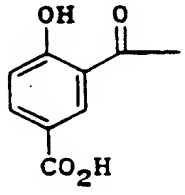

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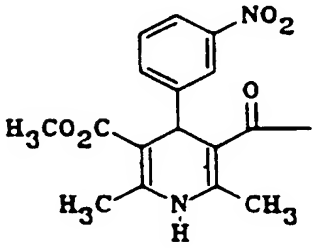

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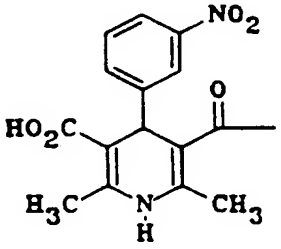

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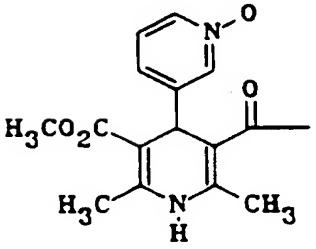

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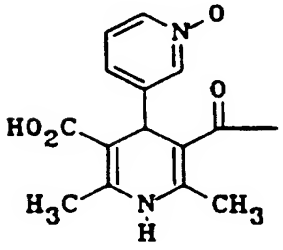

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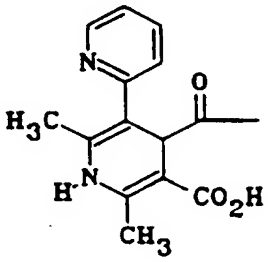

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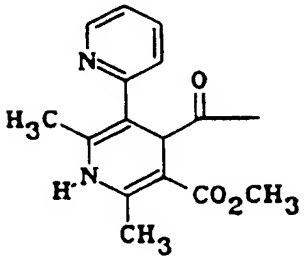

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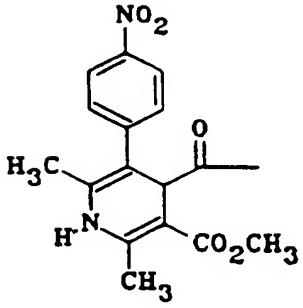

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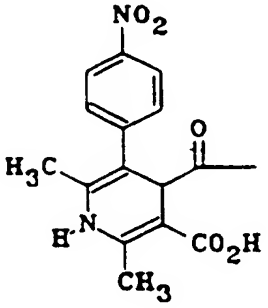
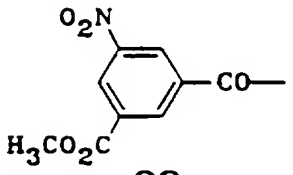


NN

FIGURE 49 (4 of 4)



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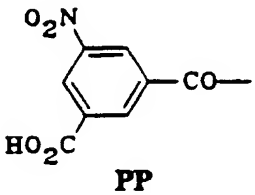

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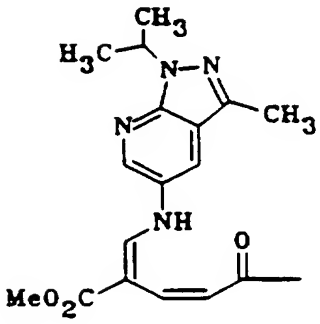

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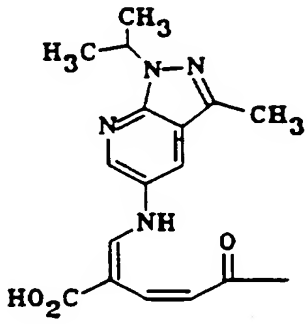

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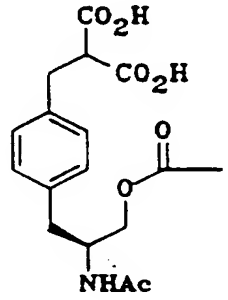


SS

FIGURE 50

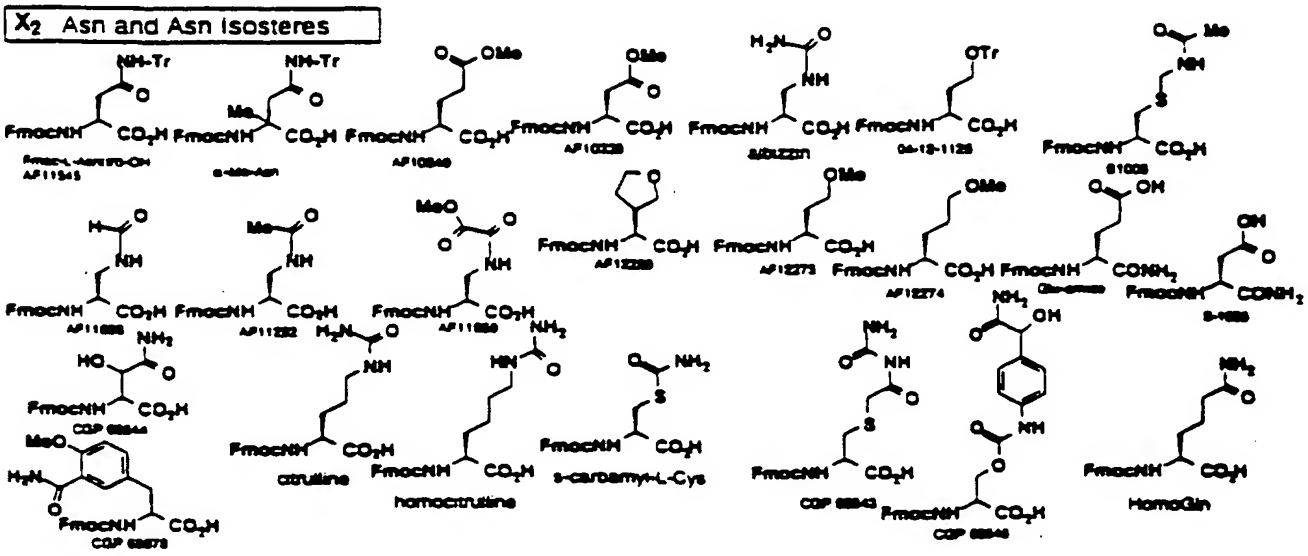


FIGURE 51

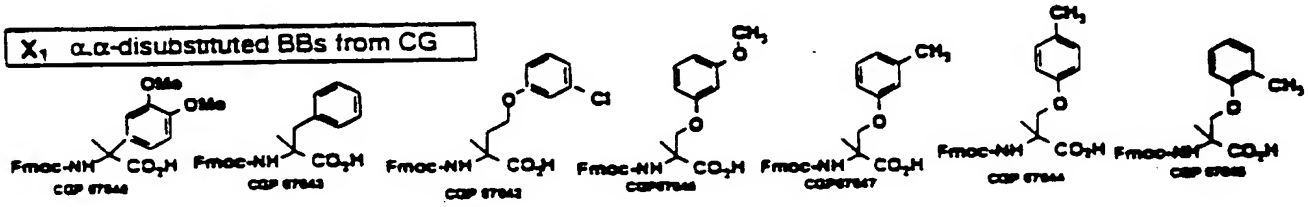


FIGURE 52

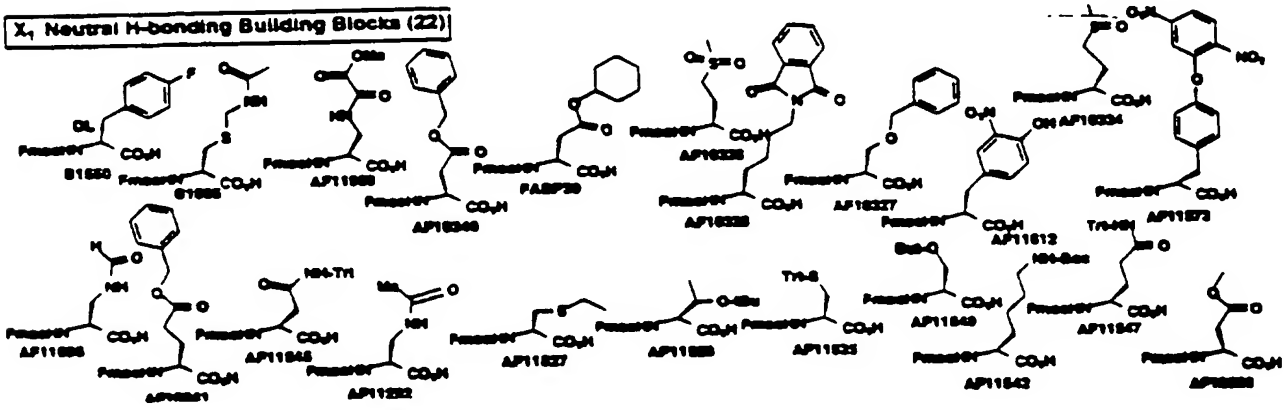


FIGURE 53

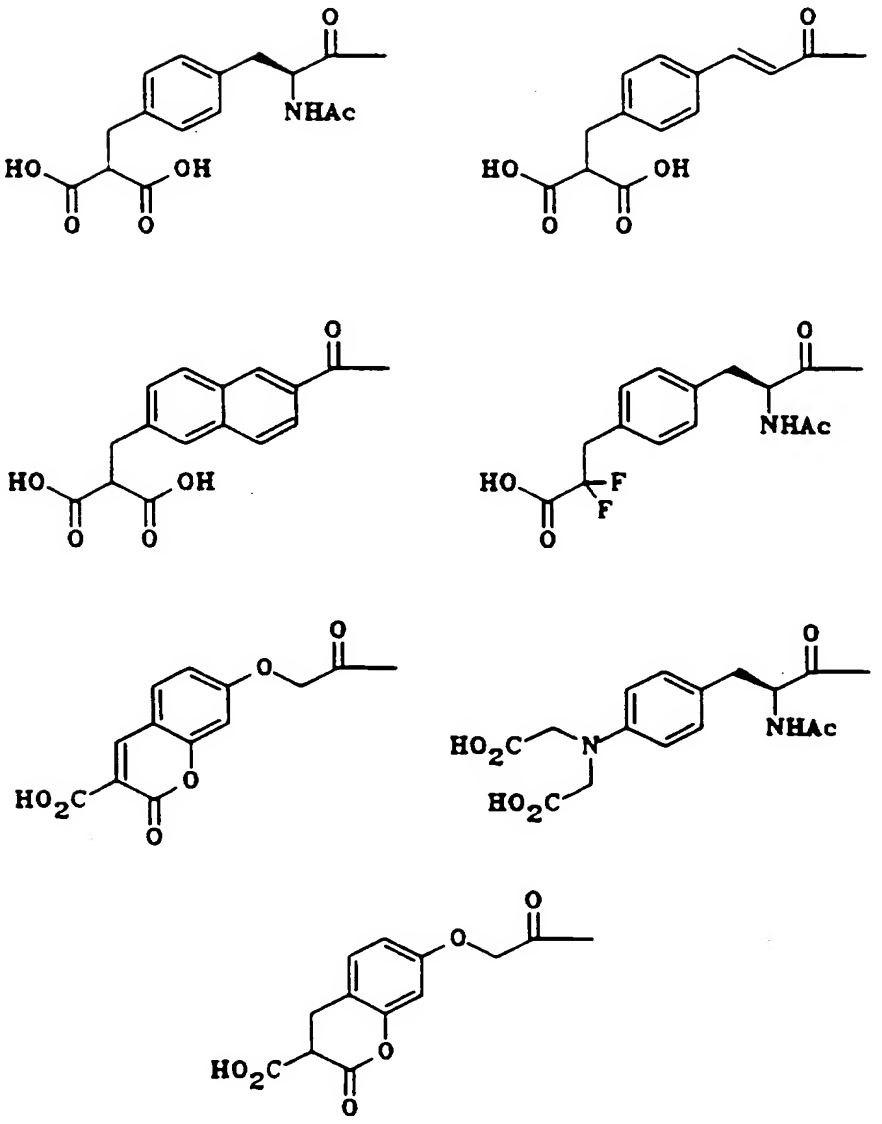
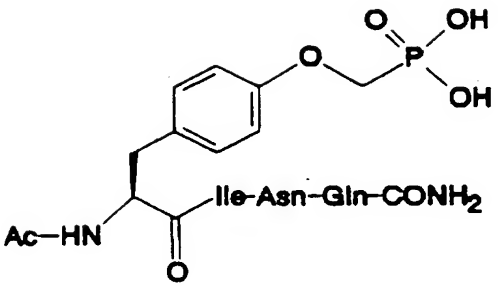
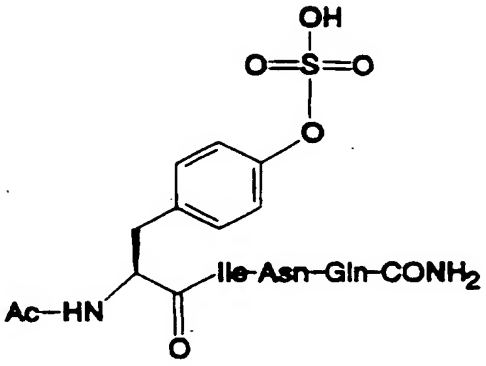
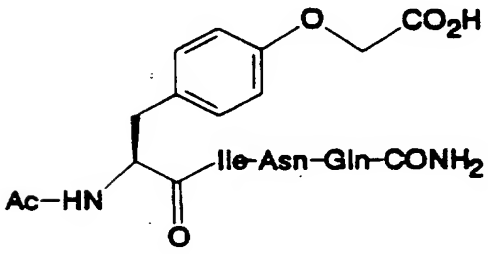


FIGURE 54







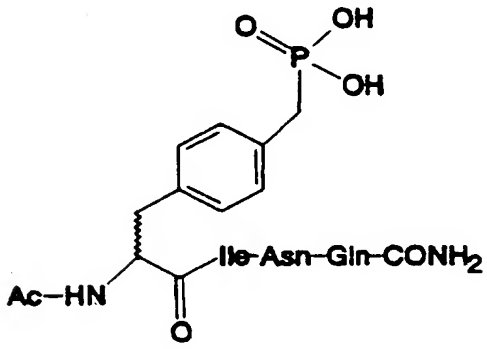


FIGURE 55(a)

Ac-F_2PmP-I-N-Q-NH_2

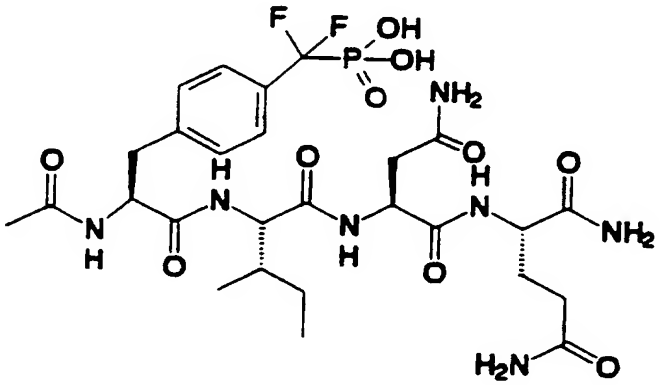


FIGURE 55(b)

Ac-E-F_2PmP-I-N-Q-NH_2

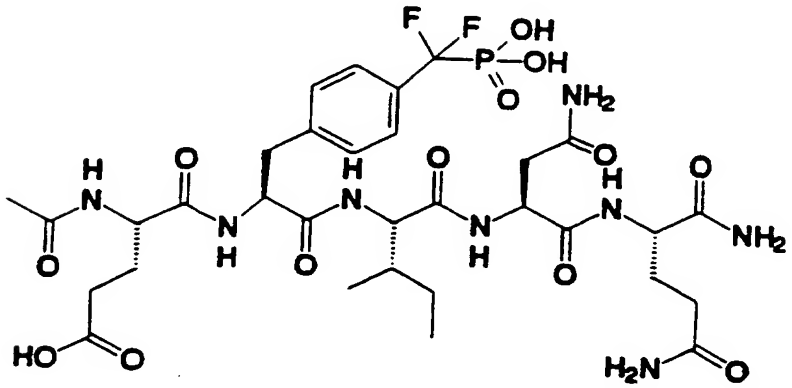


FIGURE 55(c)

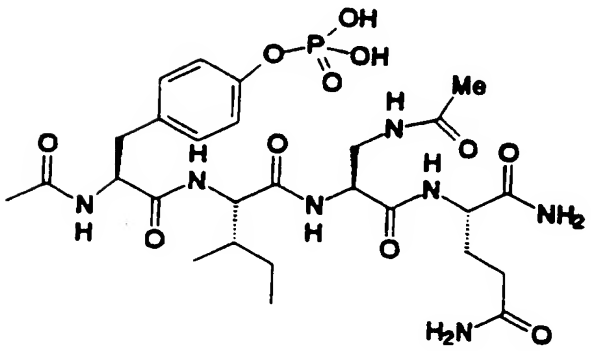


FIGURE 55(d)

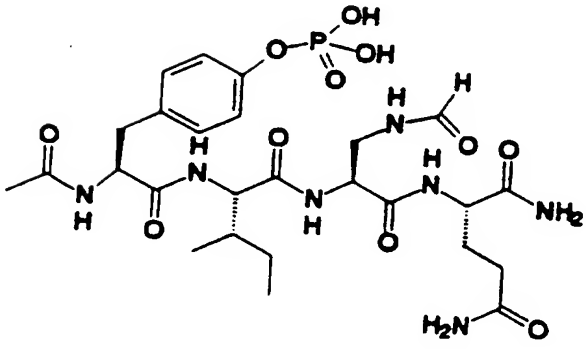


FIGURE 56(a)

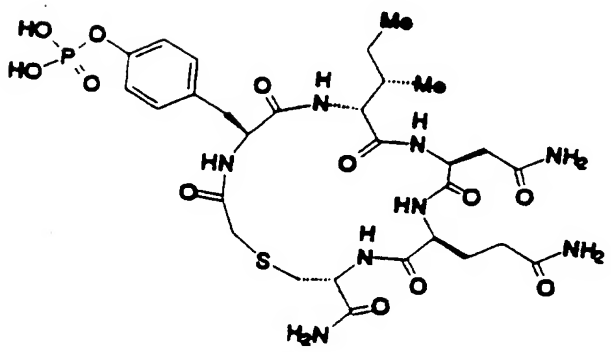


FIGURE 56(b)

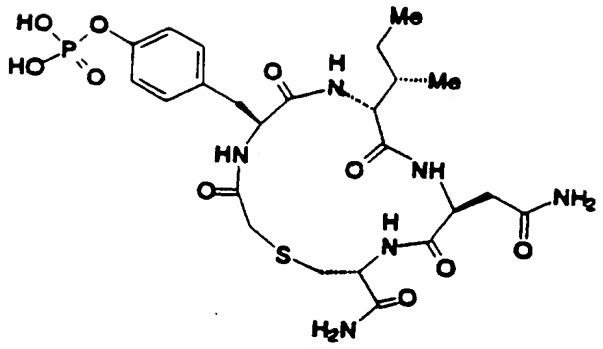
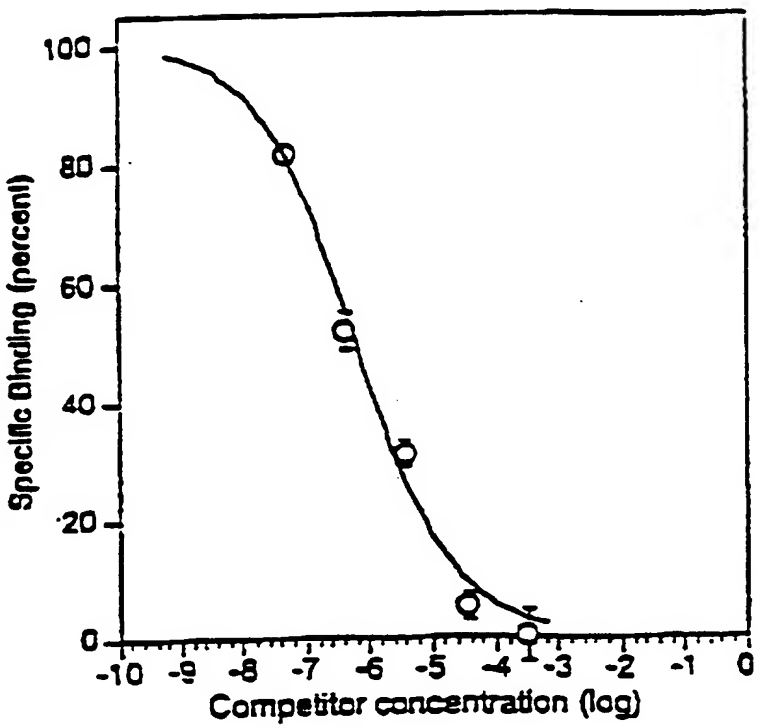
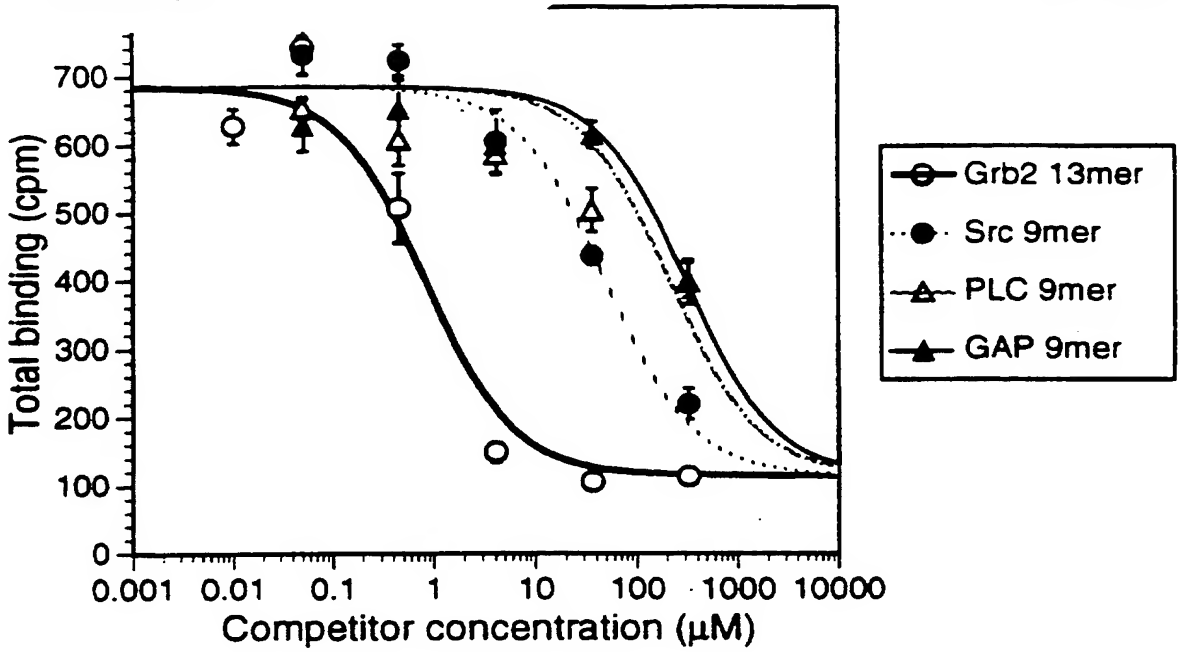


FIGURE 57


Specific Binding (percent)
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
0
Competitor concentration (log)



Competition binding assay to show specificity: testing of Grb2 SH2 with peptides known to bind to other SH2 domains

FIGURE 58

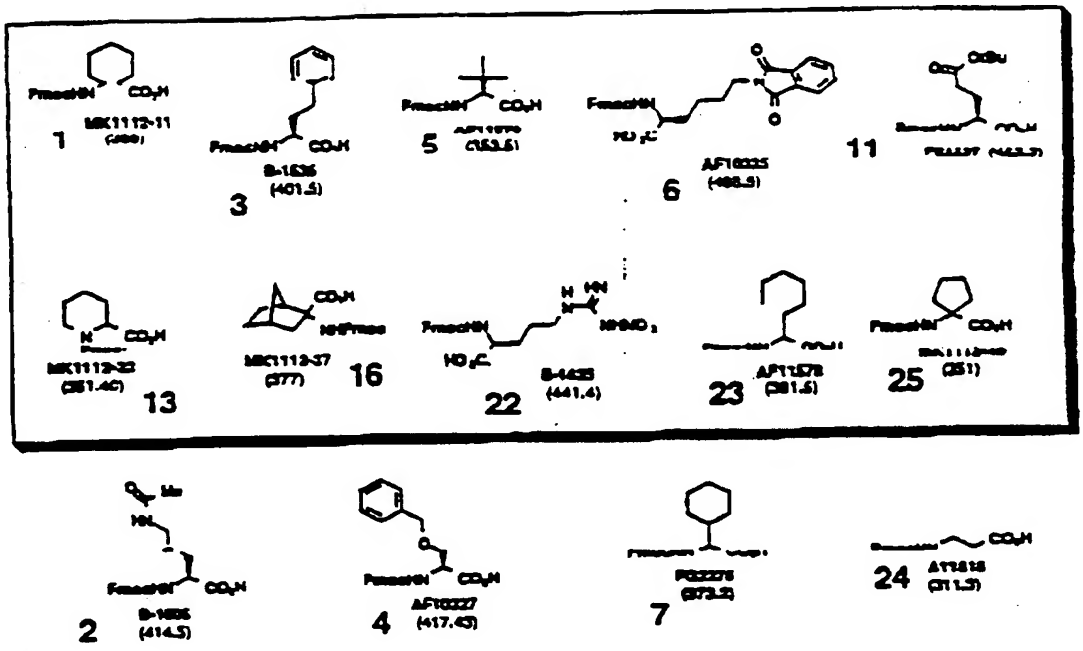
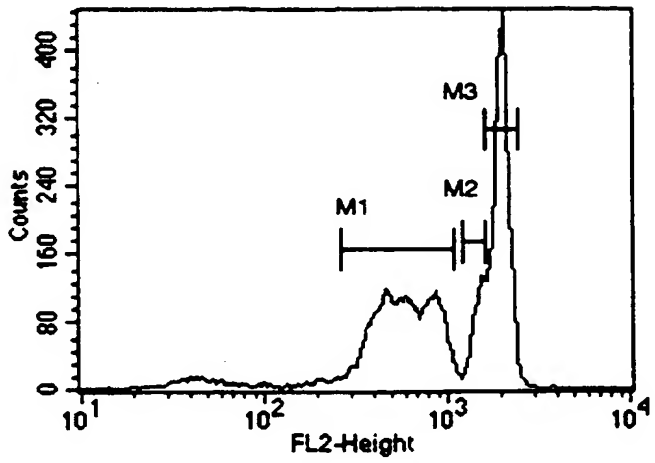


FIGURE 59

Normal analysis conditions



Affinity discriminating analysis conditions

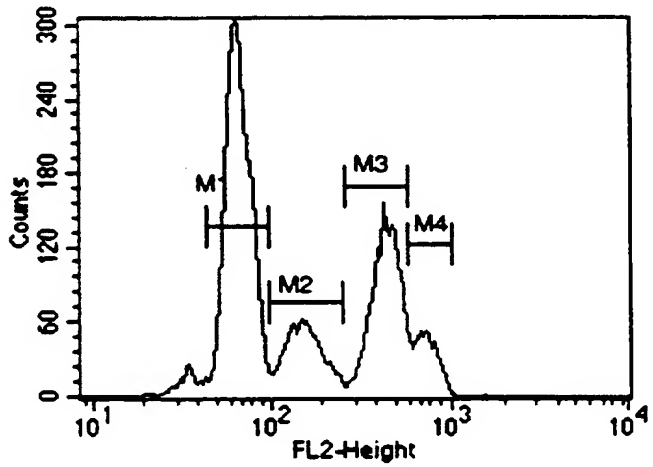


FIGURE 60

INTERNATIONAL SEARCH REPORT

Inte.. ational application No.
PCT/US96/01544

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 7/00, 7/52; G01N 33/53
US CL :435/6, 7.1; 530/326, 327, 328, 329, 330; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1; 530/326, 327, 328, 329, 330; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: SH2, phosphotyrosine, peptide, library

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 94/07913 A (WARNER-LAMBERT COMPANY) 14 April 1994, see page 11, Table1, and page 15	1-8, 26, 27 --- 9-25, 28-37
Y	CHEN ET AL. Biased Combinatorial Libraries: Novel Ligands for the SH3 Domain of Phosphatidylinositol 3-Kinase. Journal of the American Chemical Society. 1993, Volume 115, pages 12591-12592, see entire article.	9-25, 28-37
Y	SONGYANG ET AL. Specific Motifs Recognized by the SH2 Domains of CSK, 3BP2, fps/fes, GRB-2, HCP, SHC , Syk and Vav. Molecular and Cellular Biology. April 1994, pages 2777-2785, see abstract and page 2777, column 2.	9-25, 28-37

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
17 MAY 1996	31 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box P.. Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LORA M. GREEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

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